THE HEALING OF RICKETS IN RATS ON A DIET CONTAINING NEGLIGIBLE AMOUNTS OF CALCIUM AND VITAMIN D[1]

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ONE PLATE (TEN FIGURES)

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While studying the influence of large doses of irradiated ergosterol on various tissues of young rats, György ('30) observed a marked decalcification of the long bones. The area most affected was that adjacent to the actively proliferating area which the author termed the subepiphyseal spongiosa. The provisional zone of calcification showed a sharp contour, a narrow epiphyseal line and no signs of proliferation of new bone. The diet used was low in calcium. It would appear that under the influence of large doses of irradiated ergosterol calcium salts had been deposited in the growing portion of the bone, while they had been withdrawn simultaneously from the ossified part. Jones and Robson ('33) also using a diet low in calcium, obtained somewhat similar results. They found that young rats which were maintained on the Steenbock-Black rachitogenic diet ('25) from which the $CaCO_3$ had been omitted developed a rachitic-like condition of the bones. The wide uncalcified area at the metaphysis, which is typical of rickets, was not prominent, but

[1] This investigation was aided by a grant made by the Faculty Research Committee of the University of Pennsylvania. A preliminary report of this work was read before the American Institute of Nutrition meeting at Detroit on April 10, 1935.

[2] Work done while a Fellow in Orthopedic Surgery, Graduate School of Medicine.

histological studies showed a marked overgrowth of osseous tissue at the ends of the long bones. Similar results were obtained on a diet composed solely of ground whole yellow corn to which was added 1 per cent sodium chloride. Although these diets contained very little calcium and were low in phosphorus, the addition of irradiated ergosterol prevented the overproduction of osseous tissue, and bones of apparently normal architecture were obtained. The cortex, however, and spongiosal trabeculae were very thin and poorly calcified. Chemical analyses indicated that the absolute amount of ash in the femora of the animals receiving the vitamin D supplement was no greater than in the femora of the rachitic animals. Since there had been deposition of calcium salts in the metaphyseal region without increase in total bone ash there must have been withdrawal of salts from another part of the bone. Venar and Todd ('34) obtained results very similar to those of Jones and Robson.

If a deposition of calcium salts in the actively proliferating area of the bone can be coincident with their absorption from the shaft it is possible that rickets could be cured on a diet devoid of calcium. Such a study has been made in the following experiments in which were determined the effects of feeding a ration containing at most only traces of calcium to rats previously made rachitic on a modified Steenbock-Black diet (Jones, '34). The low calcium diet was fed with and without the addition of vitamin D.

EXPERIMENTAL

Several variations in the low calcium diet were used in an attempt to get a diet very low in calcium and vitamin D but otherwise adequate for growth. It was hoped that a diet sufficiently low in calcium could be obtained so that the ash from 10 gm. would not give a positive test for this element by the oxalate method. The diet finally adopted is shown in table 1.

The casein was made calcium free by the following procedure: 500 gm. of casein were allowed to stand overnight in

10 liters of approximately 0.1 N HCl. The supernatant acid solution was then removed and the casein dissolved in dilute NaOH and re-precipitated with acetic acid, washed with distilled water and again allowed to stand overnight in 0.1 N HCl. This process was repeated for 10 to 14 days; then the casein was washed and dried. Due to the possible destruction of the sulfur-containing amino acids during this treatment, as reported by Jones and Gersdorff ('34), a small amount of cystine was added. The salt mixture used is a modification of the Steenbock salt no. 40 ('23) from which the calcium phosphate and lactate were omitted and an equivalent amount of phosphorus as disodium phosphate was added. The salts

TABLE 1

Composition of low calcium diet

	per cent
Casein (acid and alkali treated)	14.00
Cystine	0.05
Straw (acid washed)	4.00
Salt mixture[1]	4.00
Lard	20.00
Lactose	15.00
Sucrose	22.45
Sucrose + wheat embryo extract	20.00
Liver extract	0.40
Carotene solution	0.10

[1] Steenbock salt no. 40 from which the calcium phosphate and lactate were omitted and an equivalent amount of phosphorus as disodium phosphate was added.

used were C.P. except the ferric citrate which was U.S.P. Roughage was furnished by 4 per cent of rye straw which was freed from calcium by washing with 0.1 N HCl. After drying, it was finely ground in a ball mill. Energy was provided by a combination of lard, sucrose and C.P. lactose. Vitamin A was supplied as an impure solution of carotene and the vitamin B factors were furnished by an 80 per cent alcoholic extract of wheat embryo and a small amount of liver extract.[3] The wheat embryo extract was evaporated on sucrose and made up to a weight equivalent to the original weight of embryo.

[3] Eli Lilly and Co. no. 343.

Several samples of this diet gave no test for calcium while in others a maximum of 0.3 mg. per 10 gm. of diet was obtained. The animals, therefore, received at most about 0.2 mg. of calcium per day as they consumed from 3 to 7 gm. of the ration. The diet contained 0.46 per cent phosphorus. Metabolism experiments showed that the animals were in a state of decidedly negative calcium balance. Individual animals were placed in cages and feces and urine collections were made for periods of 1 week. The excreta as well as the diet were analyzed for calcium. Table 2 gives the results of a few such experiments. The amount of calcium lost varied considerably with individual animals but the total calcium excreted was always greater than the intake.

TABLE 2

Calcium balances of rats on low calcium diet

RAT NO.	PERIOD [1]	DIET	CALCIUM		
			Intake [2]	Output	Loss
				mg.	*mg.*
1	1st	Low calcium + irradiated ergosterol	0.93	5.81	4.88
1	2nd	Low calcium + irradiated ergosterol	1.08	9.74	8.66
2	1st	Low calcium + irradiated ergosterol	0.90	14.55	13.65
2	2nd	Low calcium + irradiated ergosterol	0.75	28.63	27.88
3	1st	Low calcium	0.57	2.42	1.85
4	1st	Low calcium	0.78	4.68	3.90

[1] Collections made for periods of 7 days each.

[2] Calculated on basis of analysis of diet giving highest value for calcium.

Almost immediately after the rachitic animals had been transferred to this diet their intake of food became greatly reduced and several developed tetany in less than 20 hours after the change. Within 2 days nearly all the animals showed signs of tetany and a few deaths occurred. Up to this point the results were very similar to those obtained when rachitic rats were fasted. Forty-eight hours after the transference to the low calcium ration the animals were divided into three groups: The animals of group I were killed immediately by bleeding; group II was continued on the low calcium diet, and group III was given in addition 200 international units (I.U.) of vitamin D per day in the form of

irradiated ergosterol.[4] Following the 48-hour period the food consumption of groups II and III increased; signs of tetany abated or completely disappeared, and most of the animals gained weight. The death rate of the animals of group II was considerably higher than that of group III. The animals of the latter group were sacrificed at weekly or bi-weekly intervals for a period of 8 weeks. From two to four were killed at a time. Owing to the high death rate in group II it was impossible to sacrifice these animals at given intervals,

TABLE 3

Representative determinations of femur ash and serum calcium and phosphorus of rats on low calcium diet

LOT NO.	NUMBER OF ANIMALS	DAYS ON DIET	IRRADIATED ERGOSTEROL I.U.	FEMUR ASH [1]		SERUM [2]	
				Milligrams	Per cent	Ca	P
1	3	2	0	24.2	31.4	6.2	10.5
2	3	2	0	24.3	32.3	6.7	10.9
3	3	23	0	22.8	29.0	5.4	12.5
4	2	29	0	33.4	31.9	9.6	7.0
5	4	37	0	29.6	36.8	7.6	10.0
6	3	9	200	25.5	36.6	7.8	13.7
7	3	23	200	26.2	35.1	9.0	9.2
8	3	44	200	26.5	32.1	10.5	11.1
9	4	58	200	31.9	28.1	9.2	9.8

[1] Average of lot.
[2] Analysis of pooled sera.

but several lived long enough to give definite results. The blood sera were pooled and analyzed for calcium by the method of Clark and Collip ('25) and for phosphorus on the calcium-free filtrate by the method of Gunther and Greenberg ('29). The right femur of each animal was removed for ash analysis; the wrists were taken for examination by the line test technic, and the remainder of the skeleton was preserved for histological study.

In table 3 are shown the results of some of the chemical analyses which are typical of the entire series. The first two

[4] Furnished through the courtesy of Mead Johnson and Co., Evansville, Ind.

lots of animals were of group I and were killed after 48 hours on the low calcium diet. It can be observed that the calcium of the serum was low and the phosphorus was high. The next three lots were of group II and were continued on the low calcium diet for approximately 3, 4 and 5 weeks, respectively. At the end of 3 weeks the serum calcium was still low, but there was a slight increase by the fourth and fifth weeks. The phosphorus of the serum for the most part remained high. The next four lots were of group III and received irradiated ergosterol for periods of 1, 3, 6 and 8 weeks. The time in days as given in the table included the 2 days during which these animals did not receive the irradiated ergosterol. At the end of 1 week the serum calcium was only a little above that of the animals of group I. However,

TABLE 4

Average ash content of femora of all animals of each group

DIETARY GROUP	NUMBER OF ANIMALS	FEMUR ASH	
		Milligrams	Per cent
I. 48 hours on low calcium diet	20	24.5	31.5
II. Continued without irradiated ergosterol	20	28.0	33.3
III. Continued with irradiated ergosterol	19	28.6	33.7

the animals which received vitamin D for a longer period of time had a calcium level but slightly below normal. The phosphorus was more variable than the calcium and, on the whole, tended to remain high, attaining in a few cases the extremely high levels of 15 to 20 mg. per 100 cc. of serum.

The ash analyses of the femora showed slight variations. There appeared to be a general tendency toward an increase in the absolute amount of ash as well as in the percentage of ash in the femora of the animals of groups II and III as compared to group I. The increases in the ash are shown best by the averages of a larger number of animals which are given in table 4. The differences are slight but consistent, and considering the number of animals used are probably significant. No increase in the absolute amount of ash was

observed until the animals had been on the low calcium diet for at least 4 weeks. If this apparent increase in absolute amount of ash is real, the gain must have been at the expense of the ash content of other parts of the skeleton because as previously stated the animals were in a negative calcium balance. The increase in percentage of ash, on the other hand, might have been due to an actual decrease in organic matter as observed by Jones and Robson ('33).

Plate 1 shows the results of a few representative line tests on the wrists. The first photograph is that of a typical rachitic bone while the next five are of bones taken from animals which had been on the low calcium diet for 48 hours. It will be observed that there is considerable individual variation in the degree of healing[5] at this time. In the first two there is no sign of healing, in the next there is a slight but continuous line, and in the last two the degree of healing is still greater. The next two photographs are of bones taken from animals which received irradiated ergosterol for 5 weeks. In these instances the healing is complete as shown by the narrow regular epiphyseal line. Moreover, the rachitic enlargement of the end of the bone has decreased and the architecture of the bone appears normal. The extent of calcification, however, is slight as shown by the thinness of the cortex. The last two photographs are of bones taken from animals that were fed the low calcium diet without irradiated ergosterol for 30 and 57 days, respectively. In the former healing is complete as judged by the degree of calcification. However, the architecture is not entirely normal as shown by the presence of a large amount of cancellous bone and the persistence of the enlargement at the end of the diaphysis. On the other hand, the metaphysis of the radius of the rat which was on the low calcium diet for 57 days is not only completely calcified, but also appears to be as normal as those from animals which received vitamin D. Here again the cortex is extremely thin, and the entire bone shows a deficiency of calcium salts. Detailed histological studies of the

[5] As used in this paper the term healing means the degree of calcification at the rachitic metaphysis.

bones and other tissues of these animals will be reported at a later date.

From these data it appears that in the rat on a diet deficient in calcium but adequate in phosphorus, calcium salts can be transferred from one part of the skeleton to another. That is, there can be a simultaneous withdrawal of bone salts from one part of the skeleton and a deposition of these salts in another part. This transfer can be of sufficient magnitude to produce healing of rickets in rats in a few weeks and the antirachitic factor is not essential for this process. Vitamin D, however, apparently decreases the time necessary for the healing to take place. It also tends to protect the animal against the calcium deficiency as shown by the level of calcium in the blood serum and in the mortality rate of the animals on the low calcium diet.

SUMMARY

Young rachitic rats, which were transferred to a synthetic diet of adequate phosphorus content but containing at most only traces of either calcium or vitamin D, showed a marked decrease in food consumption and developed tetany within 18 to 48 hours. After 48 hours the animals were divided into three groups: group I, sacrificed; group II, continued on synthetic diet; group III, given in addition irradiated ergosterol. Serum calcium and phosphorus of group I were approximately 6 and 10 mg., respectively, and the line test indicated slight healing. The food consumption of groups II and III increased after 48 hours and tetany disappeared. Serum calcium increased while phosphorus remained high. After a few weeks both groups of animals showed complete healing of the rickets. The absolute amount of ash in the femora of the animals of groups II and III appeared to be slightly more than those of group I. Since healing of the rachitic lesions and the possible slight increase in femur ash occurred on a diet containing negligible amounts of calcium the bone salts must have been transferred from calcified portions of the skeleton to the more rachitic parts. Furthermore,

as healing took place in the animals not receiving irradiated ergosterol vitamin D is apparently not essential for this transference.

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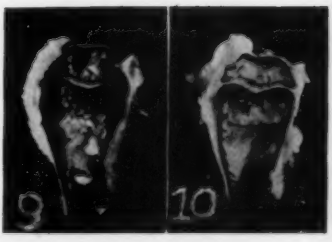

Healing of rickets in the radii of rats on a diet containing only traces of calcium.

1 Rachitic control.
2 to 6 Forty-eight hours on calcium-free diet without irradiated ergosterol.
7 to 8 Thirty-five days on calcium-free diet with irradiated ergosterol after first 2 days.
9 Thirty days on calcium-free diet without irradiated ergosterol.
10 Fifty-seven days on calcium-free diet without irradiated ergosterol.

THE EFFECT OF QUANTITATIVE UNDERFEEDING AND OF VITAMIN A DEFICIENCY ON THE TISSUE LIPIDS OF RATS FED DIETS LOW IN CHOLESTEROL

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Studies of the effect of undernutrition not only on the total quantity but also on the percentage distribution of the tissue lipids (Meyer and Schaeffer, '14; Terroine, '14) have been responsible to some degree for our concept of lecithin and cholesterol as active constituents of tissue which take part in the essential life processes of the cell, and persist even in prolonged inanition and after the supply of storage or depot fat has been exhausted.

The literature on the effect of vitamin A deficiency on tissue lipids is somewhat contradictory. Some of the papers dealing with fat deposition in young animals suggest that the effect noted may be due entirely to undernutrition while others indicate that vitamin A may exert a specific influence on tissue lipid function and distribution.

Much of the classic work on the effect of inanition on tissue lipids was carried out before the development of methods for chemical estimation of the individual lipid constituents of the tissue, or of present day technique for the feeding of animals on adequate diets made up of purified food stuffs.

[1] The material presented in this paper is taken from a thesis submitted in partial fulfillment of the requirements for the Ph.D. degree in household science in the graduate division of the University of California, Berkeley, May, 1935.

A parallel investigation of the effect on tissue lipids of 1) a limited food intake with adequate vitamin supplements and of 2) a vitamin A deficient diet, therefore appeared to be indicated. As a control it was felt that the investigation should first be carried out with diets which would give as nearly as possible 'endogenous' conditions of lipid metabolism.

We have therefore carried out:

1. A preliminary investigation of the lipid content of the easily separable tissues from the carcasses of a series of a) A deficient rats (FA–); b) litter mate controls on limited food intake, but with adequate vitamin supplements (FU); c) litter mate controls fed an adequate diet ad libitum (FN).

2. A more detailed study of the liver lipids of animals in the same three states of nutrition.

Data from this latter investigation is presented in the following paper of the series.

The basal diet used was:

Casein—baked and alcohol ether extracted	18
Crisco—(irradiated to supply 'D')	5
Agar	2
Osborne Mendel salt mixture	4
Corn starch	71

The vitamin supplements were:

A. Cod liver oil (Squibbs standardized). For the control and undernourished groups, 1 drop daily until the rats were 8 weeks of age and 2 drops thereafter; for the A deficient animals, the same amounts of oil heated to 100°C. until its A content, as determined by feeding tests, had been destroyed.

B. Dried brewer's yeast. Given as separate supplements to all groups to the extent of 4 per cent of the average food consumption until within 3 weeks of killing when 100 mg. of Harris yeast extract was substituted to guard against the immediate effects of feeding excess yeast sterols.

It should be noted that apart from the vitamin supplements this diet was sufficiently nearly sterol free to give a negative Liebermann-Burchard reaction when the alcohol ether soluble material from 1 gm. of diet was extracted with chloroform.

The lecithin content was limited to that in the yeast and cod liver oil, and the fat content held relatively low.

Grouping of experimental animals

Litters were reduced to nine, six males and three females or three males and six females, as soon as sex could be determined. Animals of the same litter and sex were divided into three groups designated as:

1. A deficient (FA), given the basal diet ad libitum with the standard yeast supplement but with heated cod liver oil.
2. Undernourished (FU), limited to an intake of basal diet which permitted only the weight gains made by the A deficient groups, but given adequate vitamin supplements.
3. Controls (FN), given the basal diets ad libitum with adequate vitamin supplements.

Plan of feeding

All animals used were placed on the basal experimental diet at weaning. The food supply of the 'undernourished' group was determined by actual weighing of the food consumed by the A. deficient partner.

Control animals of all groups were killed at the same time as the A deficient animals, i.e., after about 70 days on the experimental diet, or when the A deficient animals showed rather serious avitaminosis.

The procedures used for killing the animals and handling the tissues have been described in a previous paper (Okey, Gillum and Yokela, '34). Except as noted below the analyses reported in the tables were made by micro oxidation procedures (Bloor, '28, '29; Okey, '30). In most cases the total cholesterol determinations were also checked by a colorimetric procedure (Bloor, Pelkan and Allen, '22), and in table 1 the figures for total cholesterol reported are from colorimetric estimation.

Data from the preliminary study of pooled samples of the various tissues are given in table 1. (Data from a larger series of liver lipid analyses are given as controls in the next paper.)

TABLE 1

Lipid constituents of tissues. Preliminary series

GROUP	NUMBER OF ANIMALS	FATTY ACID	TOTAL STEROL[1] PER CENT MOIST TISSUE	LECITHIN PER CENT MOIST TISSUE	MOISTURE PER CENT
Liver					
FA-5	4	4.62	0.27	2.27	69.9
FU 5	4	3.25	0.27	2.70	70.7
FN 5	4	3.25	0.25	2.76	70.8
FA-6	4	7.47	0.32	2.44	
FU 6	4	6.38	0.40	3.47	
FN 6	4	7.45	0.32	2.54	
Hearts					
FA-5 A-6	8	1.98	0.14	1.89	73.9
FU 5 U 6	8	2.16	0.12	1.98	75.8
FN 5 N 6	8	2.26	0.13	2.04	77.4
Kidneys					
FA-5	4	5.32	0.47	1.89	70.2
FU 5	4	5.47	0.54	2.53	73.3
FN 5	4	Extract lost			71.2
FA-6	4	4.49	0.52	2.07	
FU 6	4	4.83	0.59	1.79	
FN 6	4	5.56	0.52	2.11	
Lungs					
FA-5 A-6	8	3.67	0.46	1.65	75.2
FU 5 U 6	8	3.34	0.49	2.04	78.4
FN 5 N 6	8	3.72	0.45	1.99	77.9
Spleen					
FA-5 A-6	8	2.32	0.35	1.40	73.2
FU 5 U 6	8	2.72	0.35	1.54	74.9
FN 5 N 6	8	2.66	0.33	1.54	76.3
Brain					
FA-5 A-6	8	3.44	1.28	3.55	78.7
FU 5 U 6	8	3.53	1.16	3.28	78.6
FN 5 N 6	8	3.67	1.28	3.89	79.1
Muscle					
FA-6	4	3.59	0.10	0.92	Lost
FU 6	4	6.40	0.10	0.87	74.0
FN 6	4	4.10	0.09	0.82	72.8
Skins					
FA-5	4	16.9	0.25	0.55	52.5
FU 5	4	15.3	0.26	0.54	54.2
FN 5	4	26.2	0.24	0.50	45.8
FA-6	4	17.9	0.29	0.63	
FU 6	4	21.2	0.26	0.62	
FN 6	4	25.0	0.19	0.62	
Residue					
FA-5	4	5.75	0.15	0.99	71.7
FU 5	4	4.44	0.15	1.07	68.5
FN 5	4	9.90	0.12	1.04	65.0
FA-6	4	6.48	0.14	1.12	
FU 6	4	5.23	0.13	1.08	
FN 6	4	7.50	0.11	1.14	

[1] Total sterol was determined colorimetrically in all cases except the liver, in which case the digitonide method was used.

The comparatively small differences observed in the total lipid of moist tissues of the different groups may probably be taken as a check of technique in freeing the samples from adherent body fat. From the figures for 'ground residue' which included a large proportion of bone, muscle, connective and adipose tissue, it will be seen that the 'undernourished' animals usually had even less body fat than the A deficient animals. Likewise the effect of undernutrition or vitamin deficiency was very evident in the total lipids of the skin.

Cholesterol and lecithin values show only to a very slight degree the higher proportionate retention which might be expected in the undernourished animals. That the moisture content of a number of tissues varied, usually in the direction of higher values in the control than in the vitamin deficient animals, may possibly be indicative of differences in rate of metabolism, i.e., if we may apply the hypothesis that rapidly metabolizing cells will have a higher water content than older and less active ones.

It must be emphasized that all of the data in table 1 are from the analysis of tissues of rats fed on an almost sterol and lecithin free diet, containing only 5 per cent fat and therefore may probably be taken to indicate nearly minimum variations in tissue lipids. Hence it is perhaps not strange that on the basis of Bloor's hypothesis that the lecithin/free cholesterol ratio in a tissue may be taken as an index of its metabolic activity (Bloor, Okey and Corner, '30), we found relatively little evidence of decreased cellular activity in the A deficient and undernourished animals. As a basis for distinguishing between differences in lipid content of tissues inherent in, or directly dependent upon, general condition of nutrition and not exaggerated by specifically stimulating factors in the diet, these data have appealed to us as important.

SUMMARY AND CONCLUSIONS

Data are presented from analyses of the readily separable tissues of twenty-four rats on a low sterol basal diet in the following three states of nutrition, a) vitamin A deficient, b) limited as to food intake but given adequate vitamin supplements, c) normal controls.

The percentages of lipid constituents found varied rather markedly from tissue to tissue, but apart from neutral fat, the differences observed in the lipid constituents of the individual tissues when considered with relation to the nutritional state of the animals were slight, occasionally inconsistent, but generally in the direction to be expected from earlier work on tissue lipids in undernutrition. There was no clear evidence of a specific effect of A deficiency.

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THE EFFECT OF QUANTITATIVE UNDERFEEDING AND OF VITAMIN A DEFICIENCY ON THE LIVER LIPIDS OF RATS FED DIETS WITH ADDED CHOLESTEROL

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THREE FIGURES

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In the preceding paper of this series (Gillum and Okey, '36) we reported a study of the lipid constituents of the tissues of rats, a) quantitatively underfed, b) vitamin A deficient, and c) controls. All were given a basal diet of low lecithin, cholesterol and fat content designed to give a picture of 'endogenous' tissue lipid variations in the three states of nutrition.

The present paper deals with a similar study of variations in the lipid content of tissue when one lipid constituent of the diet is increased to the point of producing a marked tissue change even in control animals. Because of the known influence of cholesterol feeding on liver lipids, and of the striking change in the liver lipids observed by one of us when cholesterol fed rats were deprived of vitamin B, study of the liver lipids of cholesterol fed rats in the three states of nutrition seemed best adapted to our purpose.

The basal diet and the plan of feeding was the same as that reported in the preceding paper, except that for approximately half of the rats from each group, 1 per cent of

[1] The data in this paper are taken from a thesis submitted in partial fulfillment of the requirements for the Ph.D. degree in household science in the graduate division of the University of California, Berkeley, May, 1935.

A preliminary report of some of this work was made at the meeting of the American Society of Biological Chemists, Detroit, April, 1935.

cholesterol was incorporated into the basal diet when the rats were about 6 weeks of age.

This meant that the animals were divided into six groups as follows:

1. FA, A deficient animals on sterol low diets.
2. CA, A deficient animals on 1 per cent cholesterol.
3. FU, quantitatively underfed animals (with adequate vitamin supplements) on sterol low diets.
4. CU, quantitatively underfed animals on 1 per cent cholesterol.
5. FN, control animals (fed the sterol low diet ad libitum with adequate vitamin supplements).
6. CN, control animals (fed the 1 per cent cholesterol diet ad libitum with adequate vitamin supplements).

The cholesterol fed animals deprived of vitamin A in the form of cod liver oil developed the typical signs of vitamin deficiency at about the same time as their partners on the sterol low diet. They, with their undernourished and normal controls, were killed after approximately 50 days of cholesterol feeding or at about 90 days of age.

The procedure for killing the rats and for making and analyzing the tissue extracts was exactly as described in the previous paper except that samples were taken from individual animals rather than pooled tissues from several animals of the same group.

Figures for total cholesterol are, however, in all cases quoted from the results of the oxidation of the digitonid rather than the colorimetric estimation. Average figures for the two sexes in the different groups of the second series of animals are reported in table 1.

Graphic comparison of all the rats as a whole are given in figures 1, 2 and 3.

It will be seen that, in general, the differences in composition between the livers of the control and the A deficient and 'undernourished' groups tended to become exaggerated when the animals were given cholesterol.

TABLE 1

A comparison of the averages of the liver lipids of vitamin A deficient, undernourished and control rats on a 5 per cent fat diet containing no added cholesterol, with those of similar animals on the same diet containing 1 per cent added cholesterol, tabulated by sex

Diet	FA–		FU		FN		CA–		CU		CN	
Sex	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Number of animals	5	5	6	2	5	6	6	5	6	5	6	5
Weight of rat grams	140	103	136	125	214	165	150	135	147	132	231	180
Moist weight liver grams	4.48	2.98	4.41	2.78	6.14	4.75	5.43	4.42	5.51	4.76	8.01	6.52
Dry weight liver grams	1.40	0.88	1.39	0.81	1.94	1.58	2.03	1.84	2.11	1.79	3.27	2.73
Moisture per cent	68.6	70.1	68.8	71.0	68.3	66.8	63.0	60.0	61.8	60.3	59.2	58.1
Fatty acids per cent moist weight	6.6	4.7	4.0	2.9	5.4	9.9	10.5	13.1	10.7	14.2	15.7	14.7
Fatty acids per cent dry weight	20.6	13.0	12.6	10.0	16.9	29.4	28.7	30.8	27.8	32.8	38.7	35.1
Fatty acid milligrams per rat	290	141	188	81	328	470	606	577	602	691	1271	966
Fatty acids milligrams per 100 gm.	206	134	133	64	153	285	417	436	397	513	548	535
Total cholesterol per cent moist weight	0.34	0.33	0.31	0.24	0.33	0.30	4.5	4.9	7.0	5.6	5.5	4.7
Total cholesterol per cent dry weight	1.08	1.07	0.98	0.83	1.06	0.92	12.4	12.4	18.5	13.1	13.4	11.2
Total cholesterol milligrams per rat	15.1	9.9	14.4	6.7	20.4	14.5	263	216	389	272	443	314
Total cholesterol milligrams per 100 gm. rat	10.8	9.6	10.3	5.2	9.5	8.7	180	166	264	202	194	174
Free cholesterol per cent moist weight	0.22	0.23	0.27	0.29	0.25	0.25	0.39	0.39	0.40	0.38	0.37	0.32
Ester cholesterol per cent moist weight	0.12	0.10	0.05	0.00	0.08	0.05	4.1	4.5	6.6	5.2	5.1	4.4
Lecithin per cent moist weight	2.8	3.0	2.9	2.1	3.1	2.9	2.4	2.7	2.8	2.7	2.8	2.8
Lecithin per cent dry weight	9.1	10.8	9.1	7.3	9.8	8.6	6.8	6.9	7.3	6.9	6.8	6.6
Lecithin milligrams per rat	127	90	135	59	189	137	133	121	148	129	220	180
Lecithin milligrams per 100 gm. rat	91	89	96	47	89	83	94	92	101	98	96	100
Lec./total cholesterol	8.5	9.2	9.2	9.0	8.9	9.6	0.63	0.61	0.40	0.53	0.54	0.62
Lec./free cholesterol	13.4	14.0	10.7	7.1	12.1	11.5	6.3	6.9	7.1	7.5	7.4	8.6

The higher moisture content of the livers of the animals on the poorer diets may probably be correlated with the lower fat and cholesterol content.

The reversal of difference between the fatty acid content of the livers of the undernourished and A deficient animals on the two basal diets may probably be explained by the very high ester cholesterol retention in the CU group. When there

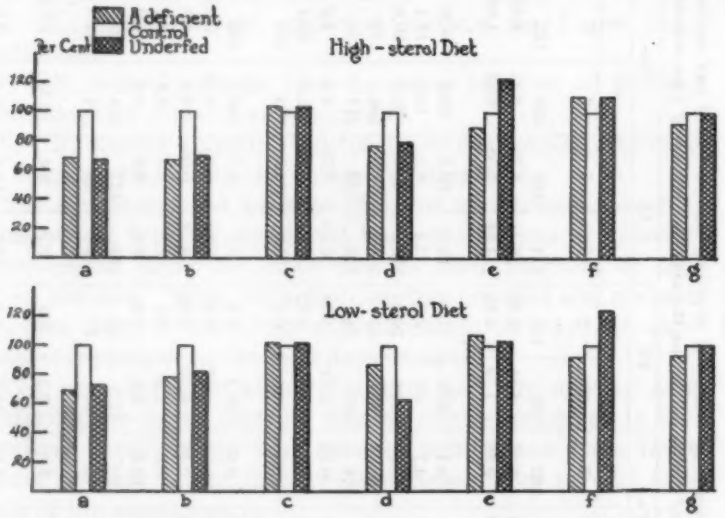

Fig. 1 A comparison of the liver constituents of vitamin A deficient, 'undernourished,' and control rats, on the basis of moist weights. The controls are taken as 100 per cent. a, weight of rat; b, moist weight of liver; c, moisture per cent; d, fatty acids, per cent moist weight; e, total cholesterol, per cent moist weight; f, free cholesterol, per cent moist weight; g, lecithin, per cent moist weight.

is no excess cholesterol in the diet, the 'undernourished' animals apparently lose an even greater proportion of fatty acid than the A deficient animals, and both these groups have far less liver fatty acid than the normal controls.

Ester cholesterol percentages for the cholesterol fed rats were, strangely enough, higher for the 'undernourished' than the normal controls. This meant not only a higher concentration (average 6.2 per cent) but almost as high an absolute

amount (average 336 mg.) in the relatively small livers of the undernourished group as in the greatly enlarged livers of the cholesterol fed controls (average 5.2 per cent and 385 mg.), and while there was a somewhat lower concentration (average 4.3 per cent) and a lower absolute amount (average 174 mg.) in the livers of the A deficient group CA, there was no such profound decrease from the control levels as that found by Okey ('33) for vitamin B deficient rats.

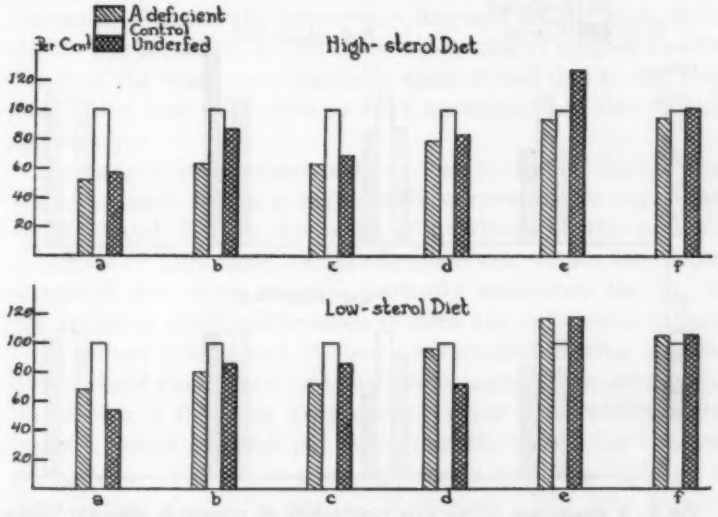

Fig. 2 A comparison of the liver constituents of vitamin A deficient, 'undernourished,' and control rats, on the basis of weight. The controls are taken as 100 per cent. a, fatty acids, milligrams per rat; b, total cholesterol, milligrams per rat; c, lecithin, milligrams per rat; d, fatty acids, milligrams per 100 gm. rat; e, total cholesterol, milligrams per 100 gm. rat; f, lecithin, milligrams per 100 gm. rat.

That there is a marked effect upon sterol storage which is associated with a condition of undernutrition appears to be evident. The actual total intake of cholesterol of the undernourished group was only slightly less (4.6 gm.) than that of the vitamin deficient animals (5.1 gm.), but was only about half that of the controls (CN group) (7.0 gm.). For the

last 2 weeks before killing the CA group averaged 1.1 gm., the CU group 1.0 gm., and the CN group 1.9 gm.

The fact that the underfed rat supplied with adequate vitamin supplements had an actual storage of 87 per cent of that of the controls with an intake only 53 per cent as great would seem to indicate some profound difference in the reaction of the two groups to the high sterol diet. It must be noted that all of these animals were on diets containing only 5 per cent

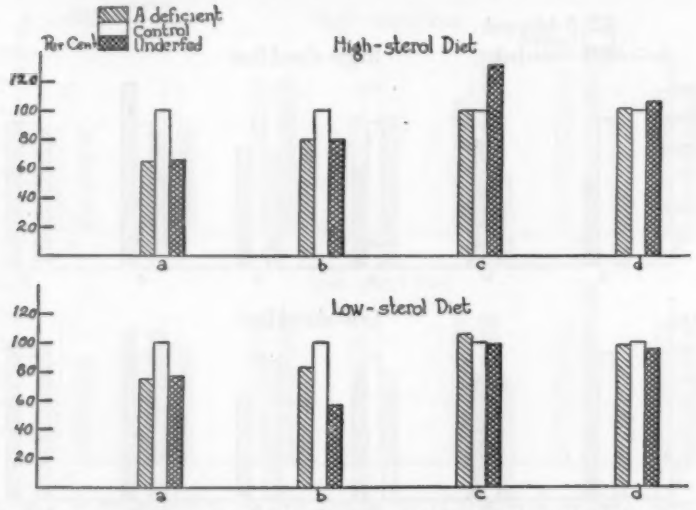

Fig. 3 A comparison of the liver constituents of vitamin A deficient, 'undernourished,' and control rats, on the basis of dry weights. The controls are taken as 100 per cent. a, dry weight of liver; b, fatty acids, per cent dry weight; c, total cholesterol, per cent dry weight; d, lecithin, per cent dry weight.

fat, and that even the normal controls stored less fat and less cholesterol ester than at higher levels of fat intake.

Cholesterol balance experiments were made with certain groups of the animals. They gave by colorimetric estimation some evidence of better cholesterol absorption in the control than in the A deficient groups. However, the dependability of colorimetric determinations of cholesterol in fecal extracts necessarily contaminated by material which interferes with the Liebermann-Burchard reaction is very doubtful. On the

other hand, many of the products of reduction of cholesterol as well as coprosterol are not quantitatively precipitated by digitonin. Hence it was felt that the reliability of methods available did not justify further work on this point.

The enlargement of the livers of the cholesterol fed controls, together with their tendency to store a great deal of cholesterol ester per rat, with a lower concentration per gram of tissue than in the undernourished animals, may be due to an inability of the liver of the latter to enlarge to meet the demands of metabolic emergency imposed by the high sterol diet. The situation in the A deficient animal suggests something of the same sort, partially ameliorated due to the poor absorption and utilization of food accompanying the vitamin deficiency.

Average lecithin concentrations in the moist livers were found to vary within a remarkably narrow range regardless of the basal diet or the state of nutrition of the animals. Analysis of individual variations observed within any group suggests that these may be partially accounted for by the unavoidable slight differences in time the rats were without food before killing and in food consumption during the day before food cups were removed from cages. The average of the entire A deficient group was 2.7 per cent which represents a decrease of 6.9 per cent from the values for the control group. This is in good agreement with the findings of Javillier et al. ('29); and of Monaghan ('32) who reports lowered phospholipids accompanying avitaminosis, and in fasting.

Phospholipid to cholesterol ratios

On the basis of the hypothesis of Bloor et al. ('30) that the lecithin/free cholesterol ratios may be taken as an index of metabolic activity, we can find no evidence of decreased cell activity in the vitamin A deficient or undernourished animals fed diet F. But when a diet containing cholesterol was ingested there was a marked decrease in the value of this ratio, and the proportionate decrease was greater in the case of the A deficient animals.

It is possible, therefore, to consider that cholesterol feeding decreases the proportion of actively metabolizing tissue in the liver, and that there is an exaggeration of this effect in the A deficient animals.

Effect of sex

It will be seen from the table that the differences between the liver lipids observed for the control animals of this series attributable to sex were in agreement with those previously reported from this laboratory (Okey, Gillum and Yokela, '34). That is, there was a higher concentration and a higher absolute amount of cholesterol in the livers of the males than of the females. That this difference became practically negligible in the case of the A deficient animals did not seem so strange in view of the sexual underdevelopment usually associated with deficiency in this vitamin. But that the differences between liver lipids in males and in females should be even greater in the undernourished groups than in the controls is somewhat difficult to explain in view of the relative infantilism of reproductive organs in the 'undernourished' animals at autopsy.

Unfortunately the pooled samples used for the analyses of liver lipids in the early groups included in this study were made up of tissues usually from two males and two females, and hence not separable by sex, and the later series did not contain enough animals to justify definite conclusions based on statistical comparison. Further investigation should prove interesting.

SUMMARY

Data are reported showing the effect of cholesterol feeding on the liver lipids of rats which were, a) vitamin A deficient, b) limited as to food intake but given adequate vitamin supplements, c) normal controls.

The size of the livers and their fatty acid content was less for the undernourished and A deficient animals both with and without cholesterol than for their respective controls.

Free cholesterol variations in liver were slight from group to group, but on both basal diets the 'undernourished' animals seemed to show a tendency to have a slightly higher concentration of free cholesterol than either of the other groups.

On diets low in cholesterol, lecithin and fat, the ester cholesterol concentration was low in the livers of all groups, and the variations of doubtful significance. When the rats were given cholesterol, the following changes were observed:

1. A lower concentration and a lower absolute amount of ester storage in the livers of vitamin A deficient than in either of the other groups. This was, however, of such a degree as to suggest poor assimilation rather than a large specific effect such as that observed in B deficiency.
2. A higher concentration and almost as high an absolute amount of cholesterol ester in the relatively small livers of the 'undernourished' animals as in the grossly enlarged livers of the controls.
3. A slightly lower phospholipid concentration was observed in the livers of the vitamin A deficient animals than in either of the other two groups on each basal diet.

The writers acknowledge their indebtedness to the board of research of the University of California for the funds which made this work possible.

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BASAL METABOLISM OF WYOMING UNIVERSITY WOMEN

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During the last few years the subject of basal metabolism has become increasingly important, and quite recently the basal metabolism of college women has claimed the attention of a number of educational institutions in various parts of the United States located at varying altitudes. Tilt ('30) published results of basal metabolism of young college women in Florida. Her subjects ranged from 17 to 25 years of age with the greater number falling in the 19- and 20-year groups. The altitude of Tallahassee, where the work was done, is 160 feet above sea level. Coons ('31) reported on the basal metabolism of Oklahoma women. Her subjects ranged from 17 to 36 years of age, with the greater number falling in the 19- to 25-year age groups. The altitude at Stillwater, where this report came from, is 870 feet above sea level.

Both of these studies reported metabolic rates well below the Aub Du Bois and Harris-Benedict standards, the usual standards used. McKay ('30) published a study on the basal metabolism of young women in Ohio. Her subjects ranged in age from 14 to 24 years, but all except five of the young women were from 14 to 18 years of age. The fact that so many of this group fell in the lower age groups makes this study less comparable with the other two, but here also all of the metabolic rates were below the Aub Du Bois standard, and the upper age group or 21 to 24 years was below the Harris-Benedict standard.

[1] The writer wishes to express appreciation to Dr. E. L. Soderlin for his interest and advice during this experiment.

Since the University of Wyoming is located at an altitude of 7148 feet and many people coming here to live have difficulty in adjusting themselves to the effects of the altitude, it seemed worth while to make a study of the basal metabolism of college women here to find out if the metabolic rate of college women was higher than at a lower altitude.

Another thing that added interest to the problem was the fact that a physician, who had practised in Ohio previously and had there, at an altitude of about 650 feet, felt the need of thyroid to speed up his metabolism, upon coming here to live was able to discontinue its use entirely.

EXPERIMENTAL SUBJECTS

The 100 young women reported in this study were all students at the University of Wyoming and were selected from a large number upon whom tests were made as being young women who were normal in every respect, as nearly as could be determined through records of their physical examinations and their health at the time the test was taken. Sixty-five per cent of the young women had lived in Wyoming all their lives, 30 per cent had lived in Wyoming more than 10 years, and the other 5 per cent came from other states, but no test was taken on a subject if she had lived at this altitude less than 6 months. All cases were discarded which showed any abnormality likely to affect the thyroid activity in any way or whose medical record contained any recent treatment for thyroid trouble. The ages ranged from 17 to 26 years, sixty-eight of the 100 falling within the 19 to 22 age groups. The weights fell within the ± 10 per cent of the Wood standards (cited by Rose, '29).

PROCEDURE

The usual procedure was followed. The young women were asked to come to the metabolism room at 7.30 or 8 o'clock without breakfast in time to rest from $\frac{1}{2}$ to $\frac{3}{4}$ hour before the test was taken, thus making the time after the last meal from 12 to 14 hours. No test was taken if the subject had not had

the usual amount of sleep the preceding night. Records of height, weight, and temperature were made, and the pulse rate was recorded a number of times during each test.

APPARATUS

A new Benedict Roth Kymographic apparatus was used, and usually three 6 minute tests were made, although occasionally if the first two tests were very regular and checked exactly, the third test was omitted. In three cases where the subject had difficulty in getting adjusted to the test as many as five or six tests were made. The lowest basal rate obtained was used, but enough tests were made to get two that corresponded almost exactly. Wilson soda lime was used and changed frequently to avoid error.

RESULTS AND DISCUSSION

The individual records, including deviations from the Du Bois (modified by Boothby and Sandiford) standards and the Harris-Benedict standards are shown in table 1.

The same information averaged according to age groups is given in table 2.

The comparison of Wyoming results with the Du Bois (modified by Boothby and Sandiford) standards is made with the original Du Bois standards in the Oklahoma and Florida studies, and the results would be less pronounced if the Wyoming tables were reduced to the Du Bois standards, since Boothby and Sandiford ('29) state that their standards are from 1 to 4 per cent lower for adults than the original Du Bois standards.

The calories per square meter of body surface per hour, using the height-weight formula, range from 33.59 to 37.87, with an average for the 100 cases of 35.61. This figure is 9.23 per cent above the Oklahoma average and 9.61 per cent above that of Florida.

The total calories per 24 hours vary from 1113 to 1748 with an average of 1368 for Wyoming women, which is 9.8 per cent above Oklahoma and 10.7 per cent above Florida. The

TABLE 1

Basal metabolism of Wyoming college women

NO.	AGE, YEARS	HEIGHT, CENTIMETERS	WEIGHT, KILOGRAMS	SURFACE AREA, SQUARE METERS	PULSE	TOTAL CALORIES PER 24 HOURS	CALORIES PER SQUARE METER PER HOUR	DEVIATION FROM STANDARD	
								Du Bois modified by Boothby and Sandiford	Harris-Benedict
1	17	161	79.0	1.82	72	1475	33.76	— 9.7	— 6.7
2	17	173	60.0	1.70	66	1489	36.48	— 2.4	+ 1.3
3	17	153	49.0	1.43	66	1361	39.66	+ 4.2	+ 2.6
4	17	166	55.3	1.60	62	1177	30.63	—18.0	—15.2
5	17	158	57.7	1.57	65	1411	37.44	+ 0.1	— 0.6
6	17	159	62.5	1.62	60	1180	30.34	—18.8	—19.5
7	18	163	52.5	1.54	68	1321	35.72	— 4.2	— 3.9
8	18	162	56.3	1.58	65	1515	39.94	+ 7.0	+ 7.6
9	18	158	59.0	1.59	75	1374	36.01	— 3.4	— 3.7
10	18	166	68.5	1.75	60	1381	32.87	—11.6	— 9.9
11	18	166	62.5	1.68	54	1596	39.57	+ 6.1	+ 8.1
12	18	175	62.0	1.74	66	1553	37.18	0	+ 4.4
13	18	171	56.3	1.65	60	1208	30.49	—18.2	—15.3
14	19	155	53.7	1.50	80	1299	36.09	— 2.1	— 5.0
15	19	159	50.0	1.49	60	1207	33.74	— 9.3	— 9.8
16	19	150	46.0	1.39	60	1220	36.57	— 1.7	— 4.9
17	19	167	62.0	1.69	60	1485	36.61	— 1.5	+ 1.2
18	19	157	60.7	1.60	62	1399	36.42	— 2.1	— 2.6
19	19	172	57.0	1.66	66	1440	36.14	— 2.2	+ 0.8
20	19	160	49.5	1.48	58	1180	33.20	—10.7	—11.7
21	19	164	53.3	1.56	60	1270	33.90	— 8.8	— 7.9
22	19	174	66.8	1.79	60	1285	29.90	—19.5	—15.8
23	19	165	58.0	1.62	50	1479	38.04	+ 2.3	+ 3.7
24	19	164	63.0	1.66	60	1354	33.98	— 8.6	— 8.0
25	19	158	59.5	1.59	70	1550	40.61	+ 8.5	+ 8.8
26	19	151	44.3	1.35	52	1488	45.92	+23.4	+17.4
27	19	155	42.0	1.35	72	1241	38.28	+ 2.9	— 1.0
28	19	169	62.5	1.70	60	1588	38.82	+ 4.6	+ 7.7
29	19	151	36.3	1.25	66	1186	39.52	— 6.3	— 0.4
30	19	163	53.5	1.55	60	1274	34.24	— 7.7	— 7.6
31	19	157	48.0	1.45	63	1163	33.41	—10.1	—11.5
32	19	168	67.5	1.75	66	1442	34.32	— 7.7	— 5.3
33	19	170	66.5	1.75	72	1263	30.06	—19.0	—16.6
34	20	163	56.3	1.59	72	1348	35.32	— 4.2	— 3.8
35	20	167	74.0	1.81	60	1658	38.12	+ 3.4	+ 5.1
36	20	160	44.0	1.41	60	1279	37.80	0	0
37	20	160	47.0	1.45	66	1303	37.42	+ 1.4	— 0.4
38	20	156	54.5	1.52	72	1376	37.71	+ 2.2	± 0.3
39	20	174	57.5	1.67	60	1749	43.62	+18.2	+22.0
40	20	163	59.7	1.62	62	1428	36.73	— 0.4	— 0.3
41	20	163	53.0	1.55	68	1347	36.20	— 1.9	— 1.7
42	20	166	53.0	1.55	57	1216	32.48	—11.9	—11.6
43	20	160	54.3	1.54	72	1248	33.76	— 8.6	— 9.3
44	20	163	62.0	1.65	64	1395	35.22	— 4.5	— 4.2
45	20	160	45.0	1.42	68	1098	32.21	—12.7	—14.7
46	20	163	56.0	1.58	60	1225	32.30	—12.4	—12.4
47	20	167	67.5	1.75	66	1416	33.72	— 7.9	— 6.5
48	20	153	44.5	1.37	60	1341	40.77	+10.5	+ 5.8
49	20	166	80.0	1.86	60	1555	34.83	— 5.6	— 4.7
50	20	166	60.0	1.66	72	1369	22.81	— 6.9	— 4.8

TABLE 1—*continued*

NO.	AGE, YEARS	HEIGHT, CENTIMETERS	WEIGHT, KILOGRAMS	SURFACE AREA, SQUARE METERS	PULSE	TOTAL CALORIES PER 24 HOURS	CALORIES PER SQUARE METER PER HOUR	DEVIATION FROM STANDARD	
								Du Bois modified by Boothby and Sandiford	Harris-Benedict
51	20	160	48.0	1.46	72	1373	39.26	+ 6.4	+ 4.3
52	20	168	65.0	1.92	64	1504	36.43	— 2.8	0
53	20	169	65.0	1.72	60	1387	33.59	— 8.9	— 7.3
54	20	153	46.5	1.40	90	1353	29.10	+ 9.1	+ 4.9
55	20	161	53.5	1.55	58	1401	37.66	+ 2.0	+ 2.2
56	20	168	54.5	1.60	72	1280	33.33	— 9.7	— 8.5
57	20	160	61.0	1.62	48	1360	34.97	— 5.2	— 5.5
58	21	156	50.0	1.47	78	1324	37.53	+ 1.7	+ 0.1
59	21	168	61.0	1.67	66	1469	36.64	+ 0.7	+ 1.2
60	21	163	69.5	1.73	60	1379	33.20	—10.0	— 9.4
61	21	157	67.0	1.66	64	1436	36.03	— 2.3	— 3.5
62	21	152	50.5	1.44	60	1259	36.43	— 1.3	— 4.6
63	21	155	56.5	1.52	64	1525	41.80	+13.0	+10.2
64	21	165	55.5	1.60	66	1486	38.69	+ 5.3	+ 6.6
65	21	165	61.0	1.65	60	1376	34.74	— 5.8	— 4.7
66	21	176	62.5	1.75	66	1511	35.96	— 2.5	+ 2.0
67	21	169	62.8	1.70	66	1495	36.65	— 0.7	+ 1.8
68	21	164	53.0	1.56	60	1209	32.80	—12.5	—11.5
69	21	160	55.0	1.55	66	1173	31.52	—14.6	—14.2
70	21	166	58.5	1.63	64	1290	32.97	—10.6	— 9.4
71	22	154	49.0	1.43	72	1114	32.45	—12.0	—14.6
72	22	165	56.5	1.60	60	1600	41.67	+12.9	+14.5
73	22	166	59.0	1.63	56	1110	28.36	—23.1	—22.0
74	22	163	64.5	1.67	60	1441	35.94	— 2.5	— 2.1
75	22	161	52.3	1.52	66	1209	33.13	—10.2	—10.4
76	22	162	48.0	1.47	60	1394	39.52	+ 7.1	+ 6.3
77	22	165	54.5	1.58	60	1345	35.45	— 3.9	— 2.7
78	22	167	63.0	1.69	56	1421	35.06	— 5.0	— 2.9
79	22	165	52.0	1.55	66	1339	35.99	— 2.4	— 1.1
80	22	165	61.7	1.67	63	1382	34.49	— 6.5	4.4
81	22	152	56.0	1.51	70	1350	37.25	+ 1.0	— 1.3
82	23	162	57.0	1.59	60	1452	38.05	+ 3.1	+ 4.4
83	23	159	52.0	1.50	66	1579	43.86	+18.0	+17.9
84	23	159	64.5	1.64	66	1535	39.00	+ 5.6	+ 5.3
85	23	177	71.0	1.85	64	1575	32.47	— 3.9	+ 1.3
86	23	162	61.3	1.63	52	1259	32.17	—12.8	—12.1
87	23	159	48.0	1.46	60	1409	40.21	+ 9.0	+ 8.3
88	23	159	47.0	1.45	72	1203	34.57	— 6.3	— 6.4
89	23	168	68.0	1.75	50	1417	33.74	— 8.5	— 5.9
90	24	160	51.0	1.51	72	1256	34.65	— 6.1	— 5.4
91	24	162	56.3	1.57	82	1548	41.08	+11.3	+12.1
92	24	165	49.5	1.51	60	1346	37.15	+ 0.6	+ 1.8
93	24	158	57.0	1.56	60	1354	36.15	— 2.0	— 1.9
94	24	170	59.5	1.68	66	1495	37.08	+ 0.5	+ 4.8
95	24	165	67.0	1.72	66	1404	34.02	— 7.8	— 5.7
96	25	161	66.0	1.68	64	1289	31.97	—12.6	—12.1
97	25	166	56.0	1.61	58	1261	32.69	—10.8	— 8.6
98	25	167	52.5	1.57	72	1361	36.12	— 0.1	+ 0.8
99	26	159	57.0	1.55	60	1214	32.64	—10.8	— 4.2
100	26	168	58.0	1.65	64	1373	34.67	— 5.2	— 1.8

calories per 24 hours per kilogram of body weight vary from 18.66 to 30.37 with an average of 24.23, which is 7.7 per cent above Oklahoma and 6.2 per cent above Florida.

The average heat production per centimeter of height varied from 6.67 to 10 calories with an average of 8.4, which is 10 per cent above either Oklahoma or Florida.

TABLE 2

Basal metabolism of University of Wyoming women averages according to age groups

NUMBER OF CASES	AGE GROUP, YEARS	HEIGHT, CENTIMETERS	WEIGHT, KILOGRAMS	SURFACE AREA, SQUARE METERS	PULSE	TOTAL CALORIES PER 24 HOURS	CALORIES PER SQUARE METER PER HOUR	DEVIATION FROM STANDARD	
								Du Bois modified by Boothby and Sandiford	Harris-Benedict
6	17	162	60.6	1.62	65.17	1349	34.72	— 7.4	— 6.3
7	18	166	59.6	1.65	64.00	1654	35.97	— 3.5	— 1.8
20	19	161	55.0	1.56	62.85	1340	35.99	— 3.8	— 3.4
24	20	163	56.7	1.59	65.12	1371	35.22	— 2.1	— 2.1
13	21	163	58.7	1.61	64.61	1379	35.76	— 3.0	— 2.7
11	22	162	56.0	1.57	62.63	1337	35.39	— 4.2	— 3.7
8	23	163	58.6	1.61	61.25	1429	36.76	+ 0.5	+ 1.6
6	24	163	56.7	1.59	67.66	1400	36.69	— 0.6	+ 1.0
3	25	165	58.2	1.62	64.67	1304	33.59	— 7.8	— 6.6
2	26	163	57.5	1.60	62.00	1294	33.65	— 8.0	— 3.0
Average		161	57.2	1.60	64.02	1368	35.61	— 3.2	— 2.5
Oklahoma						1245	32.6	—13.2[1]	—10.1
Florida		159	54.5		68.00	1236	33.4	—10.6[1]	— 9.9

[1] Du Bois.

Pulse rates varied from 48 to 90 with an average of 64 per minute, while respiration varied from 5 to 24 with an average of 13.8 per minute. No relation was found to exist between basal metabolism and either pulse or respiration.

SUMMARY

1. Basal metabolism rates were determined on a group of 100 normal college women students at the University of Wyoming, most of whom had lived in Wyoming more than 10 years. The average deviations calculated from prediction

standards are: Aub Du Bois, modified by Boothby and Sandiford, —3.18 per cent; Harris-Benedict, —2.54 per cent.

2. These metabolic rates are higher than rates found in Oklahoma and Florida under similar conditions, which would indicate that the high altitude, 7148 feet, raises the basal metabolism.

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A STUDY OF THE MAGNESIUM NEEDS OF PRESCHOOL CHILDREN

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TWO FIGURES

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The untoward symptoms following magnesium deprivation in rats observed by McCollum and Orent ('31), Kruse, Orent and McCollum ('32, '33), Kruse, Schmidt and McCollum ('33) and Greenberg and Tufts ('35) together with the findings of Duncan, Huffman and Robinson ('35) to the effect that tetany and death in calves receiving milk rations are related to the low magnesium content of the diet has led to an investigation of the magnesium needs of children. Hitherto, it has been assumed that diets consisting of a variety of foods contained sufficient magnesium, since no abnormal conditions which seemed related to a deficiency of this element have been noted. However, diets consisting of refined cereals and the generous amounts of milk advocated for children of the preschool period may contain less than the optimum amounts. In the process of refining, much of the magnesium is removed from the grain and milk is comparatively low in this constituent. Indeed, Hirschfelder ('34) has reported ten cases of low plasma magnesium associated with hyperirritability of the neuromuscular system, muscular twitchings and convulsions in patients receiving special soft diets which included considerable amounts of milk and other low magnesium containing foods.

The literature contains little information relative to the amount of magnesium needed by normal growing children. Chaney and Blunt ('25) have reported balance studies with two girls 10 and 11 years old, and Wang, Kaucher and Wing ('35), with twenty-three adolescent girls. In the former study, the magnesium retentions were related to the ingestion in a given child. There was, however, no consistent relation between the amounts ingested and retained in the two children studied. With the Wang, Kaucher and Wing studies, although the average ingestions were fairly comparable to those of Chaney and Blunt, the average retentions were somewhat less. These differences in retention may be concerned with the physical condition of the children studied, or with the fact that the diets differed in substances other than magnesium. Haag and Palmer ('28) have stressed that balanced conditions of calcium, magnesium and phosphorus are essential for normal growth.

EXPERIMENTAL PROCEDURE

In the investigation, thirty-three magnesium, calcium and phosphorus balance studies have been made with thirteen children (ten boys and three girls) between 4 and 7 years of age, receiving varying amounts of magnesium as well as calcium and phosphorus. Each balance study, with one exception, has consisted of a preperiod of 6 or 7 days to allow for adjustment to a given ingestion level, and a double metabolism period of 5 days each, thus minimizing unavoidable errors from inaccurate stool marking. The care of the children, preparation of the food and details of stool and urine collection were the same as reported in the previous study (Daniels et al., '35).

Each day's food consisted of milk, meat, eggs, potatoes, prunes, apple sauce, banana, carrots, breakfast cereal, bread, butter, and 1.5 gm. of iodized table salt, distributed among the three meals. Distilled water was used for drinking and cooking purposes. In all cases, the water used in cooking was evaporated and included in the food served. The 3.6 gm.

of a standard brand of cod liver oil plus 4 drops of viosterol twice daily, with a given amount of orange juice, and 120 gm. of canned tomato would seem to rule out any question of a vitamin D or vitamin C deficiency.

Variations in the level of magnesium were obtained by altering the amount of milk and cereals in the diets, and by using commercial whole grain bread and breakfast cereal in some of the diets and white bread and refined cereals in others. Precautions were taken to insure a constant level of magnesium in a given diet by using bread from a single loaf and cereal from the same package for an entire metabolism period for a given child. The other foods differed only in the amounts necessary to supply the protein and caloric needs of the children. The range of magnesium ingestions was not as wide as might be desired, due in part to the exigencies of other studies which were being carried on simultaneously and in part to the fact that in metabolism studies with children, diets must be planned which will be acceptable for a considerable period, since it is desirable that the same foods be prepared in the same way and taken in the same amounts during the period of study. Changes either in serving portion or preparation may result in confusion in interpretation. Furthermore, the diets used must be physiologically safe, since the children must be kept well during the study, and at the end be in as good condition as at the beginning, in so far as can be determined. Wide variations in the substances being tested, possible in animal nutrition studies, cannot be used for children for any considerable period.

The methods for the calcium and phosphorus determinations were the same as those of the former study (Daniels et al., '35). Magnesium determinations were made with filtrates from the calcium precipitates by the method described by Epperson ('28). To test the reliability of the method for the particular material under consideration a given quantity of magnesium (as anhydrous magnesium sulphate) was added from time to time to the aliquots used for analysis. Recov-

eries of from 96.1 to 100.1 per cent indicated that the method was satisfactory. Triplicate determinations were made in all cases. Results which seemed at variance were repeated.

RESULTS

Since the particular interest of the study is concerned with the magnesium needs of children, the magnesium balances have been listed in order of magnesium retentions with the corresponding calcium and phosphorus retentions and ingestions, to determine the possible influence of these on the magnesium metabolism.

In any nutrition studies with children, it is often difficult to determine to what extent the results have been modified by the nutritional condition of the child, or possibly by other factors in the diet. Studies with children differ from those with animals in that there are necessarily differences in potentialities of growth, due to different inheritances, long preperiods on a given diet are not possible, and wide variations in the substances being studied which might bring about untoward results cannot be tested. In order to determine, therefore, how much of a given substance is needed, it is desirable to study retentions with various levels of ingestion in a few children whose recent nutritional histories are known and have been controlled. Among the thirty-three balance studies reported, eight have been with one child (D.G.); with two exceptions, these were consecutive. Five balance studies with a second child (F.V.) also are listed. The dates indicate the consecutive periods for each child.

The magnesium ingestions of the children studied were between 11.3 mg. and 19.0 mg. per kilogram, whereas the magnesium retentions ranged between 0.4 mg. and 3.1 mg. per kilogram. Of these, 57.6 per cent were between 1.0 mg. and 2.0 mg. (average 1.38 mg. per kilogram), 30 per cent between 0.4 mg. and 1.0 mg. (average 0.6 mg.) and 12 per cent between 2.0 mg. and 3.1 mg. per kilogram (table 1). High retentions were not consistently associated with high ingestions, nor were low ingestions always followed by low

TABLE 1

The relation of magnesium retention to magnesium, calcium and phosphorus ingestion

CHILD	DATE	WEIGHT	MAGNESIUM				CALCIUM		PHOSPHORUS		RATIO—Ca : Mg	
			Retention per kilogram	Ingestion per kilogram	Urine per kilogram	Feces per cent of ingestion	Ingestion per kilogram	Retention per kilogram	Ingestion per kilogram	Retention per kilogram	Ingestion	Retention
		kg.	*mg.*	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*	*mg.*		
C.H.	11/14	15.8	3.1	18.2	5.4	53	53.7	12.4	73.9	9.7	2.9	4.0
D.G.	11/14	19.3	2.8	15.0	3.8	56	43.3	10.5	62.5	7.7	2.9	3.8
F.V.	11/14	15.9	2.5	18.7	4.7	62	54.1	11.7	71.6	10.0	2.9	4.7
D.G.	1/16	19.8	2.0	12.2	3.7	53	41.1	9.9	53.1	5.8	3.4	5.0
F.V.	6/11	16.8	1.8	15.6	4.4	60	49.9	10.4	66.4	7.9	3.2	5.8
J.E.	10/ 1	15.8	1.8	17.0	3.9	66	86.2	9.9	83.1	7.7	5.1	5.5
G.B.	10/22	23.7	1.7	14.5	4.1	60	59.5	10.3	65.7	5.2	4.1	6.1
F.V.	2/ 5	16.8	1.7	15.0	4.2	61	80.0	7.8	80.5	6.9	5.3	4.6
B.B.	1/16	15.4	1.6	14.3	3.8	62	50.4	6.9	63.5	8.2	3.5	4.3
F.V.	1/16	16.4	1.6	13.7	3.7	61	50.0	7.9	59.3	8.4	3.6	4.9
J.F.	2/25	19.0	1.6	11.9	3.1	60	46.6	8.4	59.2	4.6	3.9	5.3
J.F.	2/ 5	18.7	1.5	13.7	2.5	71	73.4	8.0	75.7	3.5	5.4	5.3
M.Y.	10/ 1	13.2	1.4	19.0	3.3	75	100.0	15.4	96.4	10.7	5.3	11.0
J.E.	10/22	18.6	1.4	16.7	3.7	69	81.3	11.2	78.2	8.5	4.9	8.0
P.E.	5/22	16.1	1.3	14.0	4.1	61	84.4	5.2	86.0	1.2	6.0	4.0
F.V.	12/10	16.6	1.3	13.2	4.3	58	47.4	11.1	57.2	7.7	3.6	8.5
D.G.	6/11	20.4	1.2	13.3	3.8	62	43.4	10.0	59.2	5.5	3.3	8.3
D.G.	2/ 5	19.9	1.1	12.7	3.6	63	68.7	10.2	73.1	4.8	5.4	9.3
R.S.	10/ 1	21.3	1.1	14.2	3.9	65	67.0	8.6	68.3	4.8	4.7	7.8
J.H.	3/18	15.7	1.1	14.4	3.8	66	51.2	6.0	67.5	2.9	3.6	5.5
P.E.	4/30	16.2	1.1	14.4	4.8	59	89.2	11.1	94.1	5.7	6.2	10.1
D.G.	2/25	19.9	1.0	11.3	3.4	61	44.7	7.4	55.7	3.3	4.0	7.4
R.S.	10/22	22.2	1.0	13.9	3.8	65	63.0	8.6	64.4	2.7	4.5	8.6
D.G.	5/20	20.0	0.9	11.8	3.4	64	70.2	6.9	77.3	3.8	5.9	7.7
J.E.	4/ 8	17.0	0.9	13.0	3.7	65	81.0	17.5	75.4	8.3	6.2	19.4
M.Y.	4/30	14.0	0.8	14.9	3.7	70	97.7	11.1	84.3	15.7	6.6	13.9
J.E.	3/18	16.7	0.8	13.6	3.1	71	48.7	9.9	66.5	4.4	3.6	12.4
D.G.	4/30	19.8	0.6	11.9	3.4	66	72.0	10.3	74.9	7.6	6.1	17.1
D.G.	3/18	19.9	0.6	11.6	3.2	67	41.5	6.5	56.3	1.4	3.6	10.8
D.B.	6/11	20.4	0.5	13.2	3.8	67	43.3	9.6	61.9	4.5	3.3	19.2
J.N.	12/10	14.0	0.5	14.6	3.9	70	56.0	5.8	65.5	6.4	3.8	11.6
J.H.	4/ 8	16.5	0.5	13.4	4.7	61	84.4	11.2	79.2	6.2	6.3	22.4
M.Y.	5/20	14.5	0.4	13.6	3.6	71	91.6	12.1	81.1	4.6	6.7	30.3

retentions. A consideration of D.G.'s retentions during eight metabolism periods and of F.V.'s during five (table 2) suggests that the high retentions in some of the children studied were due to previous depletion since subsequent retentions with ingestions which would seem to be adequate were lower. On the other hand, the lowest ingestions were coexistent with low retentions indicating possibly that these ingestions were

TABLE 2

The relation of magnesium ingestion to magnesium retention

CHILD	DATE	WEIGHT	MAGNESIUM			
			Intake per kilogram	Urine per kilogram	Feces per kilogram	Retention per kilogram
		kg.	*mg.*	*mg.*	*mg.*	*mg.*
D.G.	11/14	19.3	15.0	3.8	8.4	2.8
	1/16	19.8	12.2	3.7	6.6	2.0
	2/ 5	19.9	12.7	3.6	8.0	1.1
	2/25	19.9	11.3	3.4	6.9	1.0
	3/18	19.9	11.6	3.2	7.8	0.6
	4/30	19.8	11.9	3.4	7.9	0.6
	5/20	20.0	11.8	3.4	7.5	0.9
	6/11	20.4	13.3	3.8	8.3	1.2
F.V.	11/14	15.9	18.7	4.7	11.6	2.5
	12/10	16.6	13.2	4.3	7.7	1.3
	1/16	16.4	13.7	3.7	8.4	1.6
	2/ 5	16.8	15.0	4.2	9.1	1.7
	6/11	16.8	15.6	4.4	9.4	1.8

below the optimum needs. During four periods, D.G. (table 2) retained between 0.6 mg. and 1.0 mg. per kilogram. Following a considerable period of low ingestions, the effect of a higher intake was tested with the result that more was retained. F.V.'s retentions also (table 2) correlate very closely with ingestions, although at no time did he receive the low ingestions of D.G., nor were his retentions so low. Among the group with low retentions, 0.4 mg. to 0.9 mg. per kilogram, there were seven children who were receiving fairly

high ingestions. Were these lower retentions an expression of lesser need, or was the magnesium less available due to more rapid passage through the tract or some unappreciated digestive disturbance? The percentage of magnesium in the feces, based on intake, of some of this group was somewhat higher than among the children with higher retentions. Since magnesium is eliminated by way of the tract as well as through the kidney, at present there is no way of determining what proportion of the fecal magnesium is unabsorbed and what proportion is excreted magnesium.

That some of the low magnesium retentions were the result of a too low magnesium ingestion is suggested by the urinary magnesiums. D.G. excreted in the urine 3.2 mg. and 3.4 mg. per kilogram with ingestions of 11.9 mg. per kilogram or less (table 2), whereas with the higher ingestions previously tested, the urinary magnesium was consistently higher; F.V., receiving between 13.2 mg. and 18.7 mg. per kilogram and retaining between 1.3 mg. and 2.5 mg., excreted in the urine from 3.7 mg. to 4.7 mg. per kilogram. The relation of low urinary magnesium to less than optimum ingestions is also suggested by the results of Wang, Kaucher and Wing, and Chaney and Blunt. With an average ingestion of 8 mg. per kilogram (considerably less than was included in the diets of the preschool children studied) Wang, Kaucher and Wing report an average urinary output of 3.4 mg. per kilogram with an average retention of 0.4 mg. per kilogram, while Chaney and Blunt found from 2.5 mg. to 3.3 mg. per kilogram in children receiving 7.3 mg. and 10.1 mg., respectively, and retaining 1.7 mg. to 3.4 mg. per kilogram, and from 3.6 mg. to 4.0 mg. with higher ingestion levels, 10.1 mg. to 13.6 mg. per kilogram, respectively. Retentions following the higher ingestions also were higher, 2.8 mg. to 5.0 mg. per kilogram.

Assuming that high urinary magnesiums (over 3.4 mg. per kilogram) are indicative of a sufficient ingestion, then the children of the study who were retaining between 0.4 mg. and 0.9 mg. of magnesium per kilogram and were excreting over 3.4 mg. of urinary magnesium per kilogram (60 per cent of

the low retention group) were receiving sufficient for their physiologic needs. If this interpretation is correct, it must be concluded that a large proportion of the children studied had been receiving previously less than their optimum needs since 70 per cent retained 10 mg. per kilogram or more. There may be, however, other explanations not yet apparent for these low retentions.

Considering ingestions in relation to retentions and urinary magnesium, and drawing conclusions from the data at hand, it would seem that 13 mg. per kilogram of body weight is the least amount of magnesium that should be included in the diets of children of the ages studied. Further studies with other types of diets are needed to establish the validity of this conclusion. It is interesting to note, however, that the high average of magnesium in the 3000 calory diet reported by Sherman ('28) when considered in relation to the caloric intake of these children, is very comparable to this figure.

THE RELATION OF MAGNESIUM TO CALCIUM AND PHOSPHORUS METABOLISM

The interrelation of the calcium and magnesium salts postulated by the early workers seems not to have been borne out by recent research, excepting in those studies with animals where comparatively large amounts of magnesium salts either were fed or injected, and even in these the results may have been wrongly interpreted, since the introduction of an excessive amount of magnesium into the ration "resulted in such reduced palatability and lowered food intake, accompanied by such severe digestive disturbance that any specific effect of magnesium or calcium relation in the body was masked" (Elmslie and Steenbock, '29); and the untoward effects noted in conditions where the phosphorus was low or where the magnesium salts were injected may have been due to basicity, rather than to the magnesium salts per se, for with seemingly normal diets the calcium, phosphorus, and magnesium may be varied within rather wide ranges without producing visible abnormalities (Medes, '26). Furthermore,

in calves suffering from tetany, Duncan, Huffman and Robinson ('35) observed that although the serum magnesium was considerably below normal, the calcium and phosphorus were well within the normal range. Similarly, Denis and Talbot ('21) found in children suffering from various types of diseases low plasma magnesium with normal calcium values. Convulsions noted in certain cases were associated with both low magnesium and low calcium. The addition of vitamin D was without influence on the magnesium blood values, suggesting that there is little causal relationship between these elements in the blood (Duncan, Huffman and Robinson).

In studies with the human organism it was noted that the addition of magnesium lactate to the diets of two adult males was followed by a very definite increase in calcium retention in one case and a decrease in another, while magnesium retentions were significantly increased in both (Carswell and Winter, '31). Magnesium citrate also caused a marked increase in magnesium retentions, and a definite decrease in calcium retentions in the four subjects studied by Bogert and McKittrick ('22). Coons and Coons ('35), on the other hand, were unable to find any influence of variations in magnesium ingestion on the calcium and phosphorus metabolism during successive studies in a gravid human subject and conclude that "magnesium metabolism is not antagonistic to that of calcium where the intake amounts to no more than is found in ordinary dietaries."

The relation of magnesium metabolism to calcium and phosphorus metabolism in the children of the present investigation has been studied: 1) by comparing the calcium: magnesium ingestion ratios with the calcium: magnesium retention ratios, and 2) by comparing the magnesium and phosphorus retentions with the calcium retentions arranged in order of calcium retentions.

A consideration of these calcium: magnesium ingestion ratios suggests that they were not the influencing factors, either in the magnesium or calcium retentions. The ingestion ratios were between 2.9 and 6.7 whereas the retention

ratios ranged from 3.8 to 30.3, 39 per cent being between 3.8 and 5.8 inclusive, 39 per cent between 5.8 and 11.6, and 21 per cent over 12. Low retention ratios were not co-existent with low ingestion ratios, nor did high ingestion ratios result in high retention ratios. Indeed, in the children studied, there seemed to be little if any interdependence of calcium and magnesium metabolism (fig. 1). Apparently some of the children studied needed more calcium, whereas some needed more magnesium. Nor did the phosphorus metabolism appear to influence magnesium retentions; at least, there seemed to be no general trend indicating a direct relationship, whereas the relation between calcium and phosphorus retentions was very apparent (fig. 1). The relationship between calcium and phosphorus and the lack of relation of magnesium with either is further emphasized by the two children who were studied during consecutive periods (fig. 2). When the children first came to the ward, they seemingly had been receiving previously less than the optimum amount of all three metabolites for subsequently their retentions were lower. During four periods, D.G. received less than the optimum amount of magnesium since the urinary output was low and subsequently increased retentions were obtained at a higher level. Phosphorus and calcium retentions also decreased during these periods, but these obviously were not caused by low ingestions since during two periods of low retentions the ingestions were high. Only one period (June 11th) showed higher retentions of all three metabolites with higher magnesium ingestions, which suggests the possibility that under certain conditions the magnesium of the diet may be an influencing factor in calcium and phosphorus metabolism. Previously, however, equally high calcium retentions as well as high phosphorus retentions had been obtained with lower magnesium ingestions. There were fewer consecutive studies with F.V., and because of this, interpretation is difficult. The higher retentions of both phosphorus and magnesium would seem to indicate that his home diet was less satisfactory than D.G.'s. On the other hand, the magnesium ingestion may

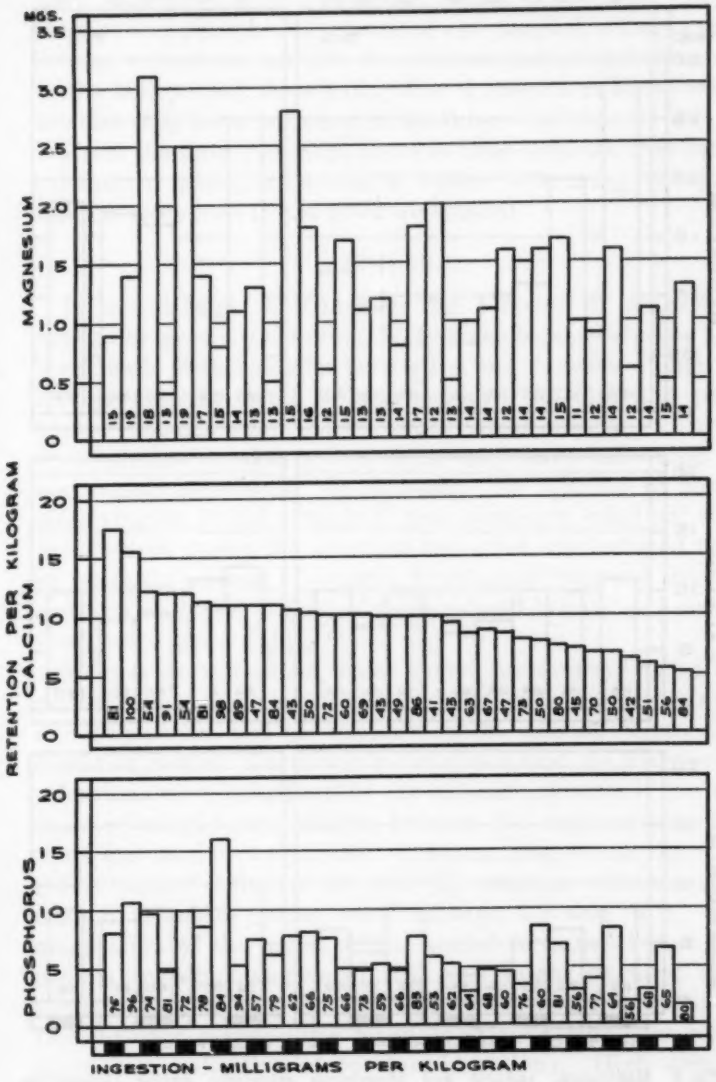

Fig. 1 Corresponding magnesium, calcium, and phosphorus retentions arranged in order of calcium retentions show no consistent interrelation.

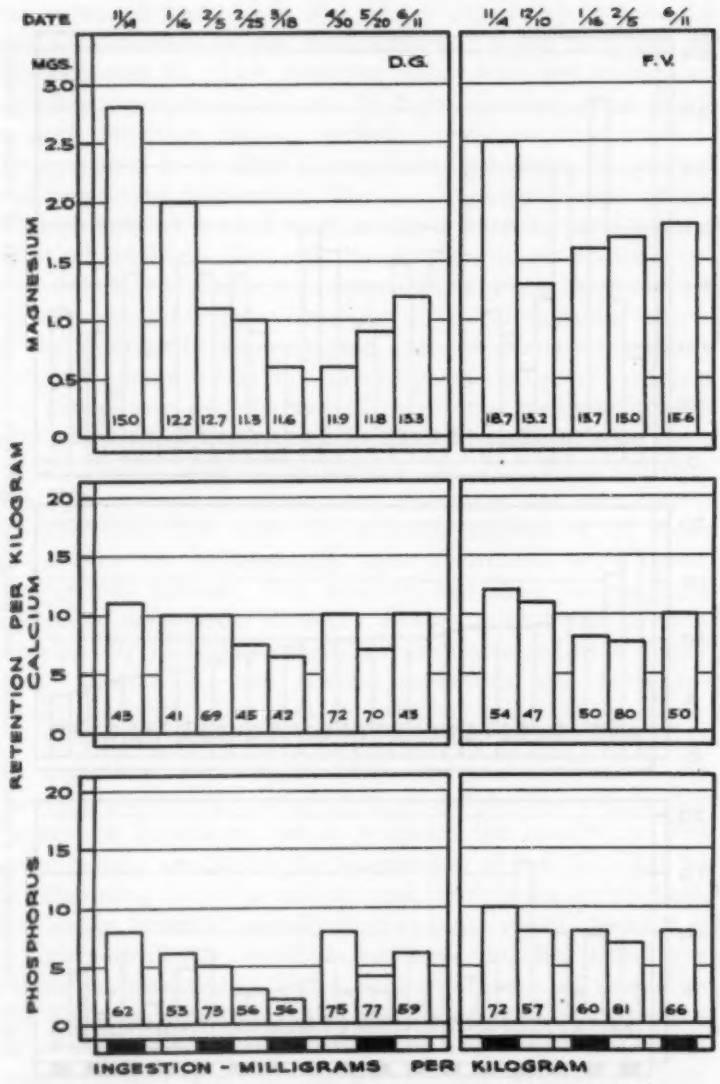

Fig. 2 Magnesium, calcium and phosphorus retentions during consecutive metabolism periods of D.G. and F.V.

have been more nearly optimum during all periods. Again calcium and phosphorus retentions run parallel, whereas magnesium retentions parallel the calcium and phosphorus only in the last period, June 11th, after 4 months at home where the diet may have been low in all three constituents.

From the data available, there is little evidence that either calcium or phosphorus was a factor influencing the magnesium retentions in the children studied.

SUMMARY

In an attempt to determine the amount of magnesium needed by growing children, thirty-three balance studies have been made with children between 4 and 7 years of age receiving various levels of magnesium. Calcium and phosphorus balance studies were made simultaneously in order to determine to what extent these may have influenced the magnesium retentions. In the study, the influence of insufficient vitamin D and vitamin C was seemingly ruled out since the children received a constant and what was believed to be an adequate amount of vitamin D obtained from cod liver oil and viosterol, and vitamin C from canned tomato and orange juice given twice daily.

Magnesium retentions, which varied from 0.4 mg. to 3.1 mg. per kilogram, were considered in relation to ingestion, urinary magnesium, and the influence of the calcium: magnesium ingestion ratios. There was no evidence that the magnesium retentions were influenced by the calcium ingestions, nor was there seemingly any relation between the magnesium retentions and calcium retentions. Calcium: magnesium ingestion ratios ranged between 2.8 and 6.7, whereas calcium: magnesium retention ratios were between 3.8 and 30.3. The magnesium of the urine, which tended to parallel the magnesium ingestion suggested that this might be used as a means of determining the sufficiency of the diet in this respect. Low urinary magnesiums when co-existent with low retentions were indicative of too low ingestions. High urinary magnesiums with low retentions were interpreted as indicat-

ing that enough had been fed and that previously the individual had been receiving a diet containing a sufficient amount, whereas high urinary magnesiums with high retentions following high ingestions indicated that the individual had been receiving previously less than the optimum amount. Seventy-five per cent of the children studied were in this group.

It was concluded tentatively that diets of children of the ages studied should contain not less than 13 mg. per kilogram of body weight. Further studies with different types of diets are needed to confirm these findings.

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STUDIES ON THE RELATION OF DIET TO GOITER

III. FURTHER OBSERVATIONS ON A GOITROGENIC DIET

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ONE FIGURE

(Received for publication November 21, 1935)

In 1933 (Levine, Remington and von Kolnitz, '33 a) we published results showing that it was possible to produce enlarged and hyperplastic thyroids in young rats fed for 5 weeks on a diet, which, while not in all respects perfectly balanced, was extremely low in iodine. This diet was identical with that employed by Steenbock to produce rickets in the rat, except that 0.2 per cent of irradiated yeast was added to prevent development of rickets. The choice of a rachitogenic diet was not deliberate on our part. We spent considerable time making iodine analyses of various foods, in an attempt to devise an otherwise adequate diet that would be sufficiently low in iodine for our purpose. It was only after obtaining discouraging results in other directions that we decided to follow up the observation of Krauss that rats on the Steenbock diet 2965 developed enlarged thyroids. Our use of this diet may have been unfortunate, in that it has led several writers to assert that the abnormal Ca : P ratio of the diet played a part in the experimental results, a question which will be discussed later in this paper.

We also showed that the addition to the diet of 3 γ per day, or less, of iodine as potassium iodide, effectively prevented the development of goiter. On this diet, the average fresh thyroid weight, per 100 gm. body weight, of 193 rats was

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53 mg. against 12 mg. for rats receiving added iodine, the dry matter content of the gland 19 per cent against 28 per cent, and the iodine content, dry basis, 0.008 per cent against 0.267 per cent. The diet contained 14 to 17 parts per billion of iodine, furnishing about 0.15 γ per rat per day.

Experiments to determine the iodine requirement of the rat (Levine, Remington and von Kolnitz, '33 b), showed that as the iodine intake was increased from 0.14 to 0.5 γ per day, the size of the gland decreased very rapidly, and when the iodine intake was further increased, the size decreased more slowly, reaching normal value at around 2 γ iodine per day. There was a definite positive correlation also between iodine intake and iodine content of the gland, and an inverse correlation between iodine intake and dry matter content. In the experiments reported, and a long series of others not as yet published, these correlations had always been found to exist so that it seems evident that under our experimental procedure, the iodine intake of the animals is indicated rather definitely by either the fresh weight, the dry matter content, or the iodine content of the gland.

The characteristic appearance of these glands on gross and histological examination, as described in the previous paper, is invariably seen in glands containing 0.08 per cent or less of iodine, dry basis, frequently but not universally in those containing up to about 0.12 per cent, but never in glands whose iodine content was as much as 0.2 per cent. Judged by these criteria, i.e., physical appearance and iodine content, it has frequently happened in our experiments that we could class glands as hyperplastic even when the enlargement was not so great as to appear, of itself, significant.

Other recent experiments on the feeding of low iodine diets have been reported by Jackson and P'An ('32), Thompson ('32, '33), Hibbard ('33), Coplan and Sampson ('35), Hellwig ('31, '35), and Sugiura and Benedict ('35). Thompson fed the Steenbock rachitogenic diet, and the same diet modified by the substitution of wheat germ for a part of the wheat gluten and by varying the calcium content. She observed by

histological methods that while iodine deficiency always resulted in hyperplasia, increasing the calcium content of the diet increased the severity of the condition. Adding iodine to the diets resulted in normal glands. Thompson's basal diet contained 30 parts per billion of iodine, and consequently probably yielded to her rats (food intakes not reported) 0.28 to 0.30 γ of iodine per day; an amount which, if fed for 35 days, according to our previously published results, should have yielded glands of double the normal size, and an iodine content of 0.0165 per cent, dry basis.

Thompson also found that the thyroid of rats fed a low iodine diet for 3 months or more underwent atrophy. In the preliminary work which led up to our selection of 35 days as the standard experimental period, we observed that rats fed our diet GP for longer periods did not show any greater degree of enlargement, and in fact, if the period were too long, the enlargement per 100 gm. body weight was apt to be less. Coplan and Sampson also observed secondary atrophy of the gland following hyperplasia on a low iodine diet. Their diet, however, used casein as a source of protein, and Osborne-Mendel salt mixture, hence was not rachitogenic.

Sugiura and Benedict, studying the effect of our diet GP on malignant growth, also observed that it was goitrogenic. However, they reported that all animals lost weight on the diet continuously until death, which occurred, on the average, in 46 days. This is so at variance with the experience of Thompson ('32), and of ourselves, that we are of the opinion that either the wheat gluten or the corn meal may have contained a toxic factor. Their animals only ate 2 gm. of food per day, whereas ours consumed 10 gm. on the average, and made weekly gains of 10 to 12 gm.

Jackson and P'An, and Hellwig, were unable to produce thyroid enlargement by low iodine diets. Analysis of the diets is not reported, but the cod liver oil concentrate employed by Jackson and P'An undoubtedly contained iodine, and other ingredients may have. Their experimental period was 7 months, and that of Hellwig 4 months, so that in both

cases they were working within the range in which Thompson and Coplan and Sampson found that secondary atrophy takes place.

A. RELATION AMONG WEIGHT, DRY MATTER AND IODINE CONTENT OF GLANDS

In support of the previously mentioned correlation among size of gland, iodine content of gland, and percentage of dry matter, we have collected and tabulated the results of all our experiments on which we have complete data, over a period of 4 years. The data consist of 125 experimental groups, totalling 1021 animals. Of this number, sixty-one groups were fed our standard goitrogenic ration or modifications of it which did not involve the addition of iodine. Nineteen experimental groups received the goitrogenic ration supplemented by varying amounts of iodine as potassium iodide. Twenty-four groups received the goitrogenic diet supplemented by various samples of dried foods which furnished known amounts of iodine. Ten groups were fed Russell's modification of the Sherman breeding diet, some of them with added iodine, and eleven groups received various purified diets which it was hoped might prove to be goitrogenic.

In figure 1 we have plotted fresh thyroid weight per 100 gm. body weight against percentage of iodine (dry basis) and against percentage of dry matter in the gland. In our previous work (Levine, Remington and von Kolnitz, '33 b) on iodine requirement in the rat, we concluded that between 1 and 2 γ per day would suffice to prevent thyroid enlargement, these daily intakes corresponding, respectively, to an iodine content in the dried gland of 0.115 and 0.177 per cent. The iodine curve in figure 1 is particularly interesting in view of this observation and also in view of the widely quoted statement of Marine that when the human thyroid contains less than 0.2 per cent of iodine (dry basis) goiter is present. Now while histological examination has revealed hyperplasia in rat thyroids containing 0.100 to 0.200 per cent of iodine, it appears from the graph that a marked increase in size does not occur until the iodine

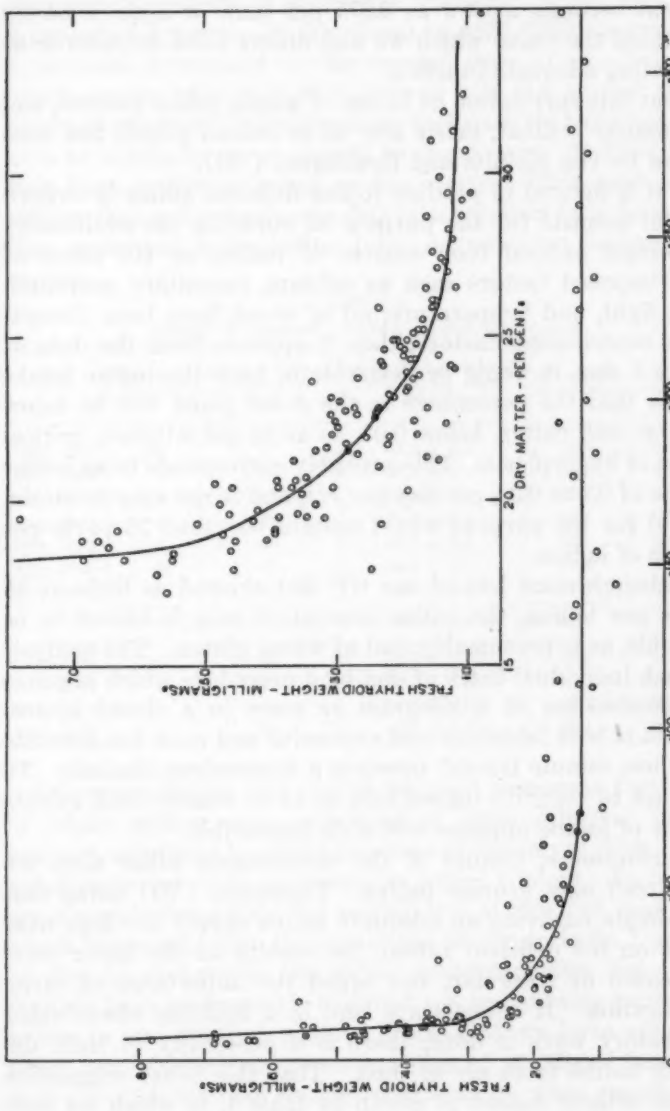

Fig. 1 Showing relationship of fresh weight with iodine content and dry matter content of thyroid glands of rats on iodine adequate and iodine deficient rations. Thyroid weight in milligrams per 100 gm. body weight.

content becomes as low as 0.075 per cent, or approximately one-third the value which we and others have considered as indicating adequate function.

That this correlation as to size of gland, iodine content, and dry matter content, exists also as to human glands has been shown by von Kolnitz and Remington ('33).

If it is desired to produce iodine deficient goiter in experimental animals for the purpose of studying the availability of certain natural food sources of iodine, or the effect of superimposed factors such as calcium, insanitary surroundings, light, and temperature, all of which have been thought to be contributing factors, then it appears from the data of figure 1 that it would be desirable to keep the iodine intake so low that the percentage in the dried gland will be below 0.05, or still better, below 0.03, so as to get within a critical range of hyperplasia. This probably corresponds to an iodine intake of 0.4 to 0.3 γ per day per rat, and is not easy to attain. A diet for the purpose would contain less than 35 parts per billion of iodine.

Although some lots of our GP diet showed as little as 15 parts per billion, the iodine content of corn is known to be variable, as is presumably that of wheat gluten. The analysis of each individual batch of diet by a procedure which requires the combustion of a kilogram or more in a closed system (which is both laborious and expensive and none too accurate for these minute traces) presents a tremendous obstacle. To attempt to repurify ingredients so as to remove such minute traces of iodine appears well-nigh impossible.

Furthermore, factors of the environment other than the diet itself may provide iodine. Thompson ('33) noted that if animals receiving an adequate iodine supply are kept near those on the deficient ration, the results on the latter were decreased or irregular, and urged the importance of strict segregation. If animals are kept in a building where other laboratory work is done, there is a possibility of their deriving iodine from air or dust. That this latter suggestion is not without reason is shown by table 1, in which we have

recorded the results of successive trials on our GP diet over a period of 4 years. In the winter of 1931–1932 we installed a chemical laboratory on the second floor of our building, which connects by an open stair-well with the animal rooms on the third floor. Subsequent to that date we have not been able to obtain as large glands as previously. Neither have they been as low in iodine or dry matter. This leads us to believe that our rats during this latter period have been getting somewhat more iodine from an unknown source than earlier.

TABLE 1

Results of successive feeding of diet GP to rats

DATE	NUMBER OF RATS	AVERAGE GAIN IN WEIGHT IN 35 DAYS	FRESH THYROID WEIGHT PER 100 GM. BODY WEIGHT	DRY MATTER IN THYROID	IODINE IN THYROID
		gm.	*mg.*	*per cent*	*per cent*
1930–1931	193	47	53.2 ± 0.9	19.0	0.0083
Feb. 1932	8	63	39.4 ± 1.7	19.1	0.0084
June 1932	9	59	23.9 ± 0.9	22.7	0.0152
Aug. 1932	9	42	24.3 ± 0.8	24.1	0.0286
Nov. 1932	8	60	24.2 ± 1.3	22.6	0.0103
Apr. 1933	9	39	30.6 ± 1.7	20.4	0.0109
Nov. 1934	15	65	29.1 ± 1.3	21.5	0.0220
KI added to furnish 3.72γ iodine per rat per day					
1930–1931	24	48	12.6 ± 0.19	28.0	0.2671
June 1932	9	55	12.1 ± 0.12	31.3	0.2000
Nov. 1932	5	42	14.3 ± 0.52	29.2	0.2828
Apr. 1933	9	33	13.2 ± 0.27	29.4	0.2600

Of course during this period we used numerous fresh lots of whole yellow corn, obtaining it from different known sources, and made repeated analyses of the mixed diets, without discovering wide variations in iodine content. In table 2 are shown iodine values for several lots of this diet, and from which it appears that the variations shown in table 1 cannot be due to changes in the iodine content of the diet itself. Neither have we been able to detect any measurable amount of iodine in our distilled water. Since 1931 we have received, dried, and ground numerous samples of sea food, however, and although we took such precautions as were possible, it

is not beyond the realm of possibility that dust from the grinding of these iodine-rich materials may have found its way to our animal and diet rooms. We agree with Thompson that if iodine intake is to be kept low enough to assure a significant and consistent degree of thyroid enlargement, the animals must be segregated from those receiving iodine supplements. We also feel that they should be segregated from chemical laboratories in which iodine in various forms may be used, and from the dust arising from the drying or grinding of iodine-rich substances.

TABLE 2

Analysis of successive lots of goitrogenic diet

DATE	IODINE	REMARKS
	parts per billion	
Jan. 1931	17	Usual rachitic diet
April 1931	20	Usual rachitic diet
	19	Rachitic diet from Fleischmann laboratories
Nov. 1931	14	With irradiated yeast 0.2 per cent
March 1932	15	With irradiated yeast 0.2 per cent
Sept. 1932	13	With irradiated yeast 0.2 per cent
Sept. 1932	20	Using commercial water-ground corn meal
Nov. 1932	14	Using corn from Ohio Experiment Station
Nov. 1932	14	Using corn from Minnesota Experiment Station
March 1933	13	
March 1933	11	Without irradiated yeast
May 1933	14	New supply of gluten

Although it may not be possible to know and control rigidly the iodine intake of rats on low iodine diets, experimental work can still be done with regard to the effect of various superimposed factors on low iodine goiter, provided, 1) the iodine deficiency is great enough to come within the critical range; 2) all animals are fed from the same batch of basal diet; 3) the handling of the animals is otherwise the same; and 4) the results as to size of gland, iodine content of gland, and dry matter content of gland, of the rats on basal diet, are in accord with the curves shown in figure 1.

With these precautions in mind, we have not found it difficult to produce glands weighing from 20 to 35 mg. per 100 gm.

body weight by feeding our GP diet for 5 weeks to 60 gm. rats: these glands will contain from 0.03 to 0.01 per cent of iodine, dry basis. If, then, one wishes to use this diet as a basis for experiments to determine the effect of varying different factors of the diet or environment, or the anti-goitrogenic value of foods, it is imperative that both positive and negative controls be run on the same batch of diet at the same time, and without variations in the environment of any group of animals.

B. EFFECT OF INCREASED IODINE IN BREEDING RATION

According to our technic, young rats of 60 gm. average weight (about 28 days old) are placed for 5 weeks on the experimental diet, at the end of which time they are killed by chloroform and the glands examined. We have previously reported (Levine, Remington and von Kolnitz, '33 a) that thirty-nine rats maintained for 9 weeks from birth on our colony ration, consisting of ground whole wheat, dried milk, dried meat scrap, and sodium chloride, yielded glands with an average fresh weight of 12.9 mg., dry matter content 23.3 per cent, and iodine content (dry basis) 0.0634 per cent. The iodine content of the colony ration varied from 47 to 72 parts per billion, but the average daily intake per rat per day during the 5 weeks experimental period was 0.74 γ. The glands were not enlarged, but the dry matter and iodine content were below normal, and some microscopic evidences of early hyperplasia appeared. From this we concluded that our colony ration was slightly inadequate as to iodine, and decided to determine what effect, if any, would result from increasing the iodine supply during the early life of the rats, and before they were placed on the goitrogenic diet.

The experimental procedure was as follows: One group of young rats was raised on the regular colony ration, and transferred to the goitrogenic ration at an average weight of 60 gm. A second group was raised on the colony ration, but potassium iodide was added to the drinking water so as to yield 1 γ of iodine per cubic centimeter, and when these rats

had reached 60 gm. in weight they also were placed on the goitrogenic ration. A third group of rats, raised on the colony ration, were placed on the goitrogenic ration supplemented by a fully adequate amount of iodine.

The results, shown in table 3, indicate that enhancing the iodine intake during the first 4 weeks of life does not enable the rats to store enough iodine to prevent the development of goiter when later deprived of this element.

TABLE 3

Effect of iodine content of breeding ration

DATE	NUMBER OF RATS	BREEDING RATION	EXPERIMENTAL RATION	DAYS ON EXPERIMENT	INITIAL AND FINAL WEIGHT	FRESH THYROID WEIGHT	DRY MATTER IN THYROID	IODINE IN THYROID (DRY BASIS)
					gm.	*milligrams per 100 gm. body weight*	*per cent*	*per cent*
October 1932	15	Stock	GP	35	64–135	25.5±0.82	22.6	0.0105
	15	Stock+KI	GP	35	59–115	23.3±0.36	24.2	0.0146
	5	Stock	GP+KI	35	60–111	14.3±0.52	29.2	0.2828

C. EFFECT OF CALCIUM

Several investigators have pointed to the high calcium content and abnormal Ca:P ratio (over 4:1) of our GP diet as being a factor, if not the predominating factor, of its goitrogenic power. Thompson ('33) reported on a diet quite similar to our own, that while thyroid hypertrophy occurred on the diet, more goiters developed, and they were larger, when the diet contained 3 per cent of added calcium carbonate than when it contained none. Hellwig ('35) added 2 per cent calcium chloride to a diet of corn meal and rolled oats (which should be very low in iodine) and found that goiter developed whether or not iodine was added to the diet, the goiters developing on a high iodine diet, however, being of the colloid rather than the parenchymatous type. An examination of the purest grade of calcium chloride we could purchase revealed 3300 parts per billion of iodine, hence the addition of 2 per cent of this salt would add approximately 0.66 γ per day to the iodine supply of the rat, which, together with the iodine

already present in the diet, might conceivably provide 0.8 to 1.0 γ per day, an amount which might be expected to remove iodine deficiency as a major factor in interpreting the experimental results. Our experience with varying the calcium content and the Ca: P ratio of our diet, is so at variance with that of these workers, that we feel that considerable more work must be done before the discrepancies can be explained, especially in view of the finding of Hibbard ('33) that sodium chloride, as well as calcium chloride, can produce the hyperplasia.

TABLE 4

Effect of varying calcium intake and Ca: P ratio

DATE	NUMBER OF RATS	DURATION OF EXPERIMENT	DIET	$CaCO_3$	Ca : P RATIO	INITIAL AND FINAL BODY WEIGHTS	FRESH THYROID WEIGHT	DRY MATTER IN THYROID	IODINE IN THYROID (DRY BASIS)	
		days		*per cent*		*gm.*	*milligrams per 100 gm. body weight*	*per cent*	*per cent*	
February 1932	8	35	GP	1	1.25	61–132	45.6 ± 2.6	18.9	0.0078	
	8	35	GP	3	4.1	65–128	39.4 ± 1.7	19.1	0.0084	
	8	35	GP	4.5	6.2	64–111	34.7 ± 2.2	19.5	0.0113	
April 1933	9	35	Rachitic	3	4.1	65– 92	36.6 ± 1.3	21.2	0.0102	Rickets
	9	35	Rachitic	1	1.25	62–104	45.9 ± 3.9	20.4	0.0087	
	9	56	Rachitic	3	4.1	67– 97	31.0 ± 1.3	22.6	0.0125	Rickets
	9	56	Rachitic	1	1.25	64–122	33.9 ± 2.7	23.6	0.0088	

In table 4 are shown the results of two experiments in varying the calcium intake and Ca: P ratio of animals on the goitrogenic ration. Certainly the evidence does not show larger glands or lower iodine when the calcium intake is increased, but rather the reverse. In the first experiment the difference in size of gland between rats fed 1 per cent of $CaCO_3$ and those fed 4.5 per cent appears to be significant (significance ratio 3:1) as does the iodine content. It may be that the $CaCO_3$ itself carried a trace of iodine. No histology was done on these glands, hence our findings cannot be considered as in complete opposition to those of Thompson until both chemical and histological examinations are made on the same group of animals.

Since completing the above experiments, we have gone back and reviewed some preliminary work done in April, 1931, and before we had adopted irradiated yeast as a source of vitamin D. In these experiments we fed the usual rickets-producing diet, but added various salts, phosphates, or phosphoric acid, or calcium chloride, in order to change both the Ca : P ratio

TABLE 5

Effect of varying Ca : P ratio and acid base ratio of the diet

MODIFICATION OF DIET	ANALYSIS			ACID OR BASIC VALUE CALCULATED	NUMBER OF RATS	TIME ON DIET	FRESH THYROID WEIGHT	IODINE IN THYROID (DRY BASIS)
	Ca	P	Ca/P					
	per cent	*per cent*		*per 100 gm. diet*		*days*	*milligrams per 100 gm. body weight*	*per cent*
None	1.51	0.35	4.3	530 cc. 0.1 N Alk.	17	35	48.6	0.0115
$CaCl_2$ instead of $CaCO_3$	1.48	0.31	4.8	....	4	35	18.1	0.0905
$MgCO_3$ instead of $CaCO_3$	0.11	0.38	0.3	....	3	35	24.6	0.0282
10.45% $Na_2PO_4 12H_2O$ added	1.40	1.43	1.0	859 cc. 0.1 N Alk.	4	35	30.2	0.0197
3.7% $NaH_2PO_4.H_2O$ added	1.38	0.98	1.4	311 cc. 0.1 N Alk.	4	35	40.4	0.0098
1.85 cc. H_3PO_4 (85%) added	1.57	0.98	1.6	37 cc. 0.1 N Alk.	4	35	49.7	0.0098
1.85 cc. H_3PO_4 (85%) 2.9 cc. HCl (36.5%)	1.58	1.23	1.3	313 cc. 0.1 N acid	4	35	53.8	0.0162

Basal diet: Yellow corn, 76; wheat gluten, 20; sodium chloride, 1; calcium carbonate, 3; plus 2 drops per rat per day of a diluted solution of oscodal.

and the acid : base ratio of the diet, and, to provide vitamin D, each rat received daily 2 drops of a diluted potent cod liver oil concentrate (Oscodal). The make-up of these diets, and the thyroid weights and iodine content of the thyroids, are shown in table 5. At the time this work was done we did not think to look for iodine contamination in chemically pure calcium chloride; when later we found an amount which was several times that contributed by the remainder of the diet,

the results are understood. It also seems to be rational to assume that the smaller glands obtained with magnesium carbonate and tri-sodium phosphate are due to iodine contamination. Unfortunately, too few rats were used in this work to draw firm conclusions, but the results indicate in rather definite manner, that if the iodine intake is kept low enough, large goiters can be produced regardless of whether the calcium content of the diet be high or low, the calcium-phosphorus ratio normal or abnormal, or the diet acid or alkaline in reaction.

TABLE 6

Effect of vitamin D on the development of goiter (date of experiment April, 1933—duration 56 days)

NUMBER OF RATS	DIET	IRRADIATED YEAST	CALCIUM CARBONATE	ADDED IODINE	BONE ASH (DRY BASIS)	GAIN IN WEIGHT	FRESH THYROID WEIGHT	DRY MATTER IN THYROID	IODINE IN THYROID (DRY BASIS)
		per cent	*per cent*		*per cent*	*gm.*	*milligrams per 100 gm. body weight*	*per cent*	*per cent*
9	Rachitic	None	3	None	30.5	30	31.0 ± 1.3	22.6	0.0125
9	Rachitic	None	1	None	46.7	58	33.9 ± 2.7	23.6	0.0088
8	Rachitic	None	3	3 γ per day	30.4	29	13.5 ± 0.3	31.9	0.2340
9	Rachitic	0.2	3	None	46.2	60	29.6 ± 1.3	23.0	0.0080
9	Rachitic	0.1	3	None	48.2	54	28.7 ± 1.8	22.7	0.0085
9	Rachitic	0.05	3	None	47.5	53	27.4 ± 1.1	22.8	0.0120
9	Rachitic	0.02	3	None	49.9	51	31.0 ± 2.0	23.1	0.0117
9	Rachitic	0.01	3	None	49.1	56	30.2 ± 2.0	22.4	0.0097

D. GOITER AND RICKETS

The Steenbock diet is rachitogenic by virtue not only of vitamin D deficiency, but also its high calcium and low phosphorus content. In the first paper of this series, we stated that either goiter or rickets could be developed independently on this diet, depending on whether it were supplemented with vitamin D or with iodine, but no data were presented in support of this point at that time. Table 6 shows the results of an experiment in which bone-ash determinations were made. The rats were maintained on the experimental diets for 8 weeks instead of the customary 5 weeks, in order that the

differences in bone-ash might be more marked. It will be noted:

1. Of the eight groups of rats fed different modifications of the diet, only that receiving added iodine had thyroids of normal size, normal dry matter content, and normal iodine content.

2. Reduction of the $CaCO_3$ in the diet from 3 per cent to 1 per cent resulted in a high bone ash, but did not lessen the severity of goiter.

3. Addition of irradiated yeast, in quantities from 0.2 per cent down to as little as 0.01 per cent of the diet, resulted in a high bone ash but also in enlarged thyroids of low dry matter and iodine content.

This yeast was claimed to have a vitamin D potency fifteen times that of standard cod liver oil, or over 1200 International units per gram. As little, therefore, as 0.01 per cent in the diet, would furnish in excess of 1 unit per day. We used 0.2 per cent in our diet but growth is but little better on 0.2 per cent than on 0.01 per cent, and significant differences in degree of goiter do not exist.

It appears, therefore, that a reduction in the calcium carbonate of the diet from 3 per cent to 1 per cent, would enable us to produce goiter without rickets even if no source of vitamin D were given, and that, above the protective dose, varying the amount of irradiated yeast up to 0.2 per cent, or an estimated vitamin D intake of 25 units per day, did not affect materially thyroid size, dry matter or iodine content, or growth.

SUMMARY

In extension of previous work on an iodine deficient diet, it is shown that in 125 experimental groups comprising 1021 rats, definite correlations exist between size and iodine content, and between size and dry matter content of the thyroid gland.

From the relationship between weight of gland and iodine content, it is deduced that experimental work on the factors which may influence low iodine goiter should be done within

the critical range, i.e., an iodine content in the gland of 0.03 per cent or less, dry basis, this probably corresponding to an iodine intake from known sources of 0.4 to 0.3 γ per rat per day, and an iodine content in the ration of 35 parts per billion or less.

Since all factors of the environment are difficult to control with certainty, it is suggested that experimental results should be evaluated only in light of simultaneous parallel experiments, and then only if the low iodine controls fall within the critical range.

The storage of iodine by young rats during the first 4 weeks of life is not sufficient to prevent goiter when placed for 5 weeks on a low iodine diet.

Varying the calcium content of the ration, its Ca : P ratio, and the presence or absence of vitamin D do not significantly affect the degree of goiter produced.

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THE ANTI-SCORBUTIC POTENCY OF REVERSIBLY OXIDIZED ASCORBIC ACID AND THE OBSERVATION OF AN ENZYME IN BLOOD WHICH REDUCES THE REVERSIBLY OXIDIZED VITAMIN

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ONE FIGURE

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Tillmans, Hirsch and Siebert ('32) have shown that the first oxidation product formed by the action of 2, 6-dichlorophenolindophenol upon decitrated lemon juice has anti-scorbutic activity and that the oxidation of the indophenol-reducing substance is reversible. Hirst and Zilva ('33) found that the reversibly oxidized product obtained by the action of iodine upon pure ascorbic acid prepared from paprika is only slightly less active than ascorbic acid which had not been oxidized. As these reports do not establish a quantitative evaluation of the anti-scorbutic potency of reversibly oxidized ascorbic acid, it seemed of interest to make a quantitative study of the physiological activity of this vitamin in its reversibly oxidized form, when administered orally and parenterally to guinea pigs. The role of this form of vitamin C in the animal body has also been investigated and will receive attention in this report.

EXPERIMENTAL PROCEDURE

Guinea pigs of market stock weighing around 250 to 300 gm. were placed upon a scorbutic diet consisting of the following: ground rolled oats, 65 parts; alfalfa leaf meal (autoclaved),

25 parts; crude casein, 5 parts; irradiated yeast, 3 parts; sodium chloride, 2 parts. This diet, supplemented with cabbage or other anti-scorbutic foods, was fed to the pigs during a short period of adjustment to laboratory conditions. When the animals were observed to make a normal rate of growth, the anti-scorbutic supplements were withdrawn, and graded doses of pure l-ascorbic acid[1] in the reversibly oxidized form were administered daily, upon a 6-day per week basis, for periods of 6 weeks or more. Negative control experiments with guinea pigs upon the basal diet were also carried out. The preparation of the oxidized form of the vitamin for administration was as follows.

Preparation of reversibly oxidized ascorbic acid

Ascorbic acid was dissolved in distilled water in concentrations that permitted the dosage to be around 1 cc. of solution. Bromine water was added until an excess was present as indicated by yellowness of the solution. Excess bromine was then removed by bubbling air through the solution. The clear solution was neutralized by adding $CaCO_3$, and the excess of the latter was removed by centrifugation. By this procedure ascorbic acid is converted quantitatively into its reversibly oxidized form and a clear, neutral solution, suitable for administration either orally or subcutaneously, is obtained.

Determination of reversibly oxidized ascorbic acid

The solution prepared above was assayed by a colorimetric method developed by one of us (Roe, '35). When ascorbic acid in its reduced form is boiled with HCl, furfural is formed. The latter is readily estimated by the aniline acetate method. Reversibly oxidized ascorbic acid does not form furfural under the same conditions, but readily yields furfural when boiled with HCl containing $SnCl_2$. The latter condition was made the basis of a method for determining reversibly oxidized ascorbic acid, which in brief is as follows.

[1] The authors wish to thank Merck and Company, Rahway, New Jersey, for a liberal supply of pure l-ascorbic acid which was used in this investigation.

One cubic centimeter of solution containing about 0.1 mg. of reversibly oxidized ascorbic acid is placed in a test tube and 1 cc. of 30 per cent HCl containing 1 per cent $SnCl_2$ is added. A standard for comparison is prepared by placing in a test tube 1 cc. of a solution containing an amount of xylose that will produce furfural equivalent to 0.1 mg. of ascorbic acid in its reduced form and adding 1 cc. of the HCl-$SnCl_2$ mixture. (Xylose is used as a standard because it keeps indefinitely when dissolved in saturated benzoic acid.) The two tubes are boiled in a water-bath for 10 minutes, being placed in the bath and removed therefrom simultaneously. After cooling the tubes, 2 cc. of glacial acetic acid and 3 cc. of an alcoholic aniline solution, containing 1 part of redistilled aniline to 2 parts of alcohol, are added to each tube. The tubes are shaken thoroughly to mix the contents and comparison is made in a colorimeter after waiting 5 minutes for maximum color development.

In our experiments reversibly oxidized ascorbic acid was prepared and assayed as indicated above each day, and administration was carried out as soon as conveniently possible after analysis of the solution. In each analysis a control test for reduced ascorbic acid was made. This was done by boiling 1 cc. of the vitamin solution with HCl not containing $SnCl_2$. Under such conditions furfural is not formed from reversibly oxidized ascorbic acid and hence no color is obtained when aniline acetate is added. A solution known to be free of reduced ascorbic acid, and whose quantitative composition had been ascertained with a high degree of accuracy, was thus obtained.

This method of preparing and feeding the oxidized ascorbic acid was adopted for several reasons. Bromine was used because complete oxidation was assured and excess bromine is readily removed by aeration. The solution was neutralized with $CaCO_3$ because at the resulting pH the oxidized vitamin remained stable. Analyses of the oxidized ascorbic acid solution 3 hours after preparation did not reveal any losses. The colorimetric method of analysis permitted a very exact determination of the amount of vitamin present in the solution fed.

To get control data, experiments were carried out with the vitamin in its reduced form. A freshly prepared solution of the same ascorbic acid, dissolved in distilled water and neutralized with $CaCO_3$, was administered each day to a group of guinea pigs to obtain data for comparison with the results of feeding the oxidized form.

The dosage of vitamin was based upon body weight in accordance with the method of assay of vitamin C adopted by Dann and Cowgill ('35). The pigs were weighed weekly and the dosage was changed at the time of each weighing. The average weight of each group under study was taken as the basis for the calculation of the new dosage.

TABLE 1

Feeding experiment using ascorbic acid in the reduced and the reversibly oxidized forms

GUINEA PIG NO.	ASCORBIC ACID			WEIGHT		
	Form administered	Method of administration	Dose per 100 gm. of body weight	Initial	6 weeks	8 weeks
			mg.	*gm.*	*gm.*	*gm.*
1	Oxidized	Subcutaneous	0.25	275	255	
2	Oxidized	Subcutaneous	0.25	278	240[1]	
3	Oxidized	Subcutaneous	0.25	265	237	
4	Oxidized	Subcutaneous	0.25	257	182[1]	
5	Reduced	Subcutaneous	0.25	278	458	
6	Reduced	Subcutaneous	0.25	290	462	
7	Reduced	Subcutaneous	0.25	255	388	
8	Reduced	Subcutaneous	0.25	247	401	
9	Oxidized	Subcutaneous	0.50[2]	255	365	413
10	Oxidized	Subcutaneous	0.50	275	363	409
11	Oxidized	Subcutaneous	0.50	262	320	378
12	Oxidized	Subcutaneous	0.50	248	355	418
13	Oxidized	Oral	0.50	265	315	390
14	Oxidized	Oral	0.50	250	374	470
15	Oxidized	Oral	0.50	248	328	385
16	Oxidized	Oral	0.50	268	378	455

[1] Guinea pigs nos. 2 and 4 died 2 and 4 days, respectively, before the end of the 6-week period.

[2] Five-tenth milligram per 100 gm. was administered to pigs 9 to 16, inclusively, for 6 weeks and then the dose was raised to 1 mg. per 100 gm.

Results of feeding experiments

The results of these experiments are shown in table 1 and figure 1. When 0.25 mg. of reversibly oxidized ascorbic acid per 100 gm. of weight was injected subcutaneously into four guinea pigs daily, the animals lost weight and showed some clinical signs of scurvy. Two pigs survived for 6 weeks upon this regime and the other two died during the fifth week.

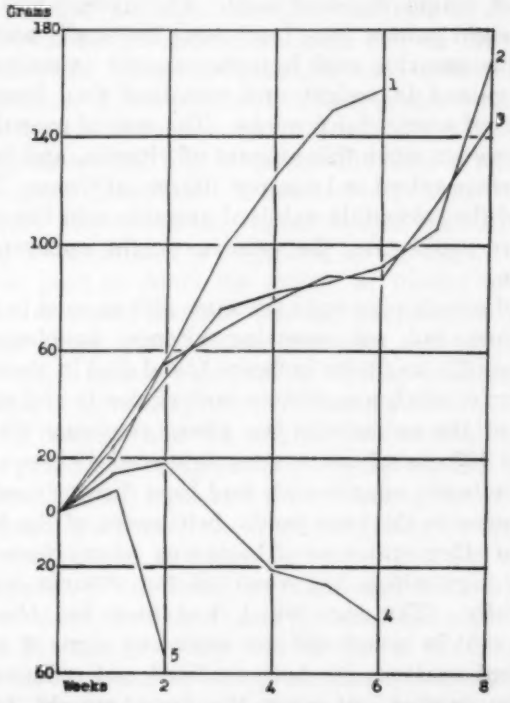

Fig. 1 Composite growth curves showing the cumulative weekly gains of guinea pigs receiving daily doses of ascorbic acid per 100 gm. of body weight as follows: 1, 0.25 mg. of l-ascorbic acid in its reduced form, subcutaneously; 2, 0.5 mg. of reversibly oxidized l-ascorbic acid for 6 weeks, and 1 mg. of the same preparation for the last 2 weeks, orally; 3, same as 2 except that the oxidized vitamin was administered subcutaneously; 4, 0.25 mg. of reversibly oxidized l-ascorbic acid, subcutaneously; 5, negative controls with basal diet only.

Four guinea pigs upon the same diet receiving the same dosage of ascorbic acid in the reduced form by subcutaneous injection grew at a normal rate and remained free from symptoms of scurvy. These experiments showed that the reversibly oxidized form of ascorbic acid has protective properties, but that its potency is considerably less than that of the vitamin in its reduced form.

A dosage of 0.5 mg. of reversibly oxidized ascorbic acid per 100 gm. of weight was next used. The vitamin was administered to eight guinea pigs, four being fed orally and four receiving the ascorbic acid by subcutaneous injection. These animals gained in weight and remained free from clinical symptoms of scurvy for 6 weeks. The rate of growth was not rapid, however, upon this amount of vitamin, and the dosage was therefore raised to 1 mg. per 100 gm. of tissue. Upon this dosage of the reversibly oxidized ascorbic acid the pigs grew at a more rapid rate, the gain in weight apparently being maximum.

Control guinea pigs upon the same diet as used in the above experiments, but not receiving vitamin supplements, lost weight rapidly, as shown in figure 1, and died in about 3 weeks from scurvy which was obvious both clinically and at autopsy.

Some of the animals in the group receiving 0.5 mg. and 1 mg. per 100 gm. of tissue were autopsied 48 hours after the oxidized vitamin supplements had been discontinued. Slight hemorrhages in the knee joints, brittleness of the bones and teeth, and other evidences of beginning scurvy were observed in those pigs which had received the vitamin supplement parenterally. The pigs which had been fed the oxidized ascorbic acid by mouth did not show any signs of scurvy.

Chemical analyses for both oxidized and reduced ascorbic acid were carried out upon the liver, muscle, brain, and adrenal glands of the animals receiving 0.5 mg. and 1 mg. of the oxidized vitamin per 100 gm. of tissue. Our colorimetric method was used to determine both the oxidized and reduced vitamin C content and the analyses for the reduced vitamin content were checked with the Tillmans' 2, 6-dichlorophenol-indophenol method, as modified by Bessey and King ('33).

The results of these chemical analyses showed that there was practically no vitamin C stored in the tissues of the animals receiving reversibly oxidized ascorbic acid, either orally or by subcutaneous administration, the data obtained in these studies being essentially within the blank values of the methods used.

Reductase in blood

In view of these findings it became desirable to study the relation of reversibly oxidized ascorbic acid to metabolism. It was obvious that either the oxidized form is an intermediate in the metabolism of reduced ascorbic acid or else the oxidized vitamin is converted into its reduced form by the tissues. The first assumption seemed highly improbable since it was found that the oxidized form has a much lower potency than the reduced form. The second hypothesis seemed more likely and experiments were planned to determine its validity.

Blood was used to study the ability of tissues to reduce oxidized ascorbic acid because this tissue maintains a more normal metabolism after removal from its natural environment than other tissues obtained by slicing or maceration. Ascorbic acid, dissolved in 0.9 per cent NaCl solution, was oxidized with bromine and the solution was neutralized with $CaCO_3$, the procedure being entirely similar to that used to prepare the oxidized vitamin in the feeding experiments. Potassium oxalate was used as an anticoagulant for the blood and pooled samples of human blood were used for most of the experiments. Whole blood, blood plasma, and washed red cells were mixed with oxidized ascorbic acid and placed in an incubator at 38°C. for 3 hours. At the end of the incubation period the blood mixtures which contained red cells were centrifuged. Five cubic centimeter portions of the plasma, or the centrifugate from the red cells, were diluted with 20 cc. of water, 1 cc. of glacial acetic acid was added, and the mixture was titrated with 2, 6-dichlorophenolindophenol.

The results of these experiments are shown in table 2, which includes representative data from a larger series. Human

blood, guinea pig blood, and rat blood were found to have the capacity to reduce reversibly oxidized ascorbic acid. In experiments 1 and 2, human plasma and serum gave characteristic indophenol reduction values after incubation at 38°C. with oxidized ascorbic acid for 3 hours. In experiment 3 human red cells were washed ten times with 0.9 per cent NaCl

TABLE 2

Effect of blood on reversibly oxidized ascorbic acid. Indophenol titration of oxalated blood after 3 hours incubation at 38°C. with 1 mg. of reversibly oxidized ascorbic acid per cubic centimeter. The titrations were made upon 5 cc. of fluid, except in experiment no. 9

EXPERIMENT	2, 6-DICHLOROPHENOLINDOPHENOL TITRATION		
	Total	Blank	Amount due to reduced form of the vitamin
	cc.	cc.	cc.
1. Human plasma	13.4	2.8	10.6
2. Human serum	8.8	1.8	7.0
3. Centrifugate from human erythrocytes washed ten times with 0.9 per cent NaCl solution	10.1	2.8	7.3
4. Human plasma plus 1 mg. NaF per cubic centimeter	10.9	2.9	8.0
5. Human plasma plus 2 mg. NaF per cubic centimeter	2.9	2.9	0
6. Human plasma plus 5 mg. NaF per cubic centimeter	2.9	2.9	0
7. Human plasma, control on 4, 5 and 6	13.0	2.9	10.1
8. Human plasma, heated 5 minutes at 100°C.	3.4	3.4	0
9. 50 cc. tungstic acid filtrate, 1: 10 dil.	1.5	1.0	0.5
10. Human plasma plus 1 cc. $CHCl_3$	14.5	3.4	11.1
11. Human plasma control on 10	16.8	3.4	13.4
12. Human plasma plus 1 cc. ethyl ether	11.3	1.8	9.5
13. Human plasma control on 12	11.2	1.8	9.4
14. Guinea pig plasma	14.0	2.5	11.5
15. Rat blood, erythrocyte centrifugate, 0.9 per cent NaCl	9.0	2.5	6.5

solution and then physiological salt solution containing oxidized ascorbic acid was added in a quantity equal to the amount of plasma removed. After 3 hours incubation at 38°C. the red cell centrifugate showed a characteristic reduction of indophenol.

Experiments were carried out to determine whether the substance in blood which reduces oxidized ascorbic acid is

an enzyme. Plasma was mixed with sodium fluoride, chloroform, and ethyl ether. Sodium fluoride in a concentration of 2 mg. per cubic centimeter of plasma completely inhibited the reduction of oxidized ascorbic acid. Chloroform only interfered slightly and ether had no effect upon the reduction. After plasma had been heated for 5 minutes on a water-bath at 100°C. it did not show any capacity to reduce the oxidized vitamin. Obviously heating coagulates the proteins of blood plasma and does violent damage to the system, but a heating experiment was performed since destruction by heat is one of the criteria to determine the presence of an enzyme. The removal of proteins from whole blood with tungstic acid gave a filtrate with a negative capacity to reduce oxidized ascorbic acid.

The above results show that there is a substance in blood which has the ability to reduce oxidized ascorbic acid and they seem to indicate clearly that this substance is an enzyme.

Experiments with urine gave entirely negative results. There is no substance in urine having the power to reduce oxidized ascorbic acid under the conditions of our experiments with blood.

DISCUSSION

The data of this report show that ascorbic acid is approximately one-fourth as potent in its oxidized form as in its reduced form when judged by the promotion of growth and the prevention of clinical signs of scurvy in the guinea pig. Oral administration of the reversibly oxidized vitamin is much more effective in preventing the onset of scurvy than is the parenteral method. The pigs given the oxidized vitamin by subcutaneous injection showed beginning signs of scurvy in 48 hours after withdrawal of the vitamin, while the pigs given the oxidized vitamin by mouth, autopsied 1 week after discontinuance of the vitamin, did not show signs of scurvy. These results are opposite to those observed by Grollman and Firor ('34) and Hou ('35) with the vitamin in its reduced form, the latter authors finding the reduced form of vitamin C more potent when administered parenterally. The greater

protection of the oxidized vitamin, when administered by mouth, is believed to be due to the more delayed introduction of the vitamin into the tissues. In an experiment in which reversibly oxidized ascorbic acid was mixed with intestinal contents of a guinea pig and placed at 38°C. it could not be demonstrated by indophenol titration that the oxidized vitamin is reduced by intestinal contents.

The oxidized vitamin fed by us is apparently the first oxidation product of ascorbic acid. When assayed by our colorimetric method of analysis, it was found to be quantitatively converted into the reduced form of ascorbic acid by HI, and HCl-$SnCl_2$, and by treatment with H_2S 80 per cent is returned to the reduced form. The product has an $[\alpha]_D^{27}$ value of + 52.5° in acid solution with a pH of 0.90.

The results obtained by us are not in quantitative agreement with the report of Hirst and Zilva ('33), who found that oxidized ascorbic acid "is only slightly less active than ascorbic acid which had not been previously oxidized." Hirst and Zilva prepared their compound by oxidation with iodine, while our preparation was an oxidation product of bromine, but it is believed our compound is the same as that fed by Hirst and Zilva, as the regeneration data and specific rotations of the two compounds are in fair agreement.

The explanation of the anti-scorbutic activity of oxidized ascorbic acid is undoubtedly that this compound is converted into its reduced form by a substance present in the blood. This substance is believed to be an enzyme because it is inactivated by sodium fluoride, heating, and deproteinization with tungstic acid.

The finding of a reductase for oxidized ascorbic acid in blood is of considerable interest. From this observation it seems probable that ascorbic acid exists in the blood only in the reduced form. This enzyme may perhaps have functions other than maintaining vitamin C in its reduced form and might participate in other ways in the oxidation-reduction system of the animal body.

SUMMARY

1. Data have been obtained which show that reversibly oxidized ascorbic acid has approximately one-fourth the anti-scorbutic potency of ascorbic acid in its reduced form when administered to guinea pigs.

2. Reversibly oxidized ascorbic acid is more potent anti-scorbutically when given orally than when administered subcutaneously.

3. Ascorbic acid is not stored in the tissues of guinea pigs, either in the oxidized or reduced form, when the reversibly oxidized form is administered in amounts as high as 1 mg. per 100 gm. of body weight per day.

4. The anti-scorbutic effect of reversibly oxidized ascorbic acid is due to an enzyme in blood which has the power to reduce the reversibly oxidized form of this vitamin.

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RESULTS OF FEEDING VARIOUS LEVELS OF SOIL CONTAINING BERYLLIUM TO CHICKENS, DOGS AND RATS[1]

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The experimental work reported in this paper was originally initiated for the purpose of investigating the report that the ingestion of soil by animals in certain counties in northern Michigan was deleterious to their health and well-being. The toxicity of the soil was thought to be due to its beryllium content. The results are of additional interest because of the absence in the literature of data on the effect of ingestion of soil by animals.

Guyatt, Kay and Branion ('33) demonstrated that 'beryllium rickets' was produced in rats which received a normal diet by the addition of small amounts of beryllium carbonate. The diet contained optimum amounts of calcium and phosphorus and a generous allowance of cod liver oil. Jacobson ('33) found that the Steenbock diet 2965 modified by the substitution of a chemically equivalent amount of beryllium carbonate for calcium carbonate produced a disease in rats which was anatomically indistinguishable from rickets. Fabroni ('33), however, stated that the intravenous injection of beryllium hydroxide into rabbits had no demonstrable toxic or harmful effect despite the storage of the colloid in the reticulo-endothelial system. Sobel, Goldfarb and Kramer ('35) eliminated the factor of intestinal absorption by studying the mechanism of calcification under in vitro conditions

[1] Published with the permission of the director of the experiment station as Journal Article No. 241 (n.s.).

and concluded that there was a local disturbance in calcification in beryllium rickets.

In view of the above reports in the literature concerning the toxicity of beryllium, feeding experiments were conducted to ascertain the possibility of the existence of a natural beryllium toxicosis and to observe also the effect of feeding high levels of soil to various animals. For purposes of comparison some of the animals were fed beryllium carbonate with the control ration.

EXPERIMENTAL

Samples of the suspected soil were analyzed qualitatively by the method of Chamot and Mason ('31), quantitatively by the method of Washington ('30), and spectrophotometrically.

The mechanical analysis of the soil, which was of the silty loam type, showed that it contained 54.0 per cent fine sand, 21.6 per cent silt and 24.4 per cent clay. Approximately 33 per cent of the clay was colloidal.

The mean value found from ten quantitative analyses of the suspected soil was 0.615 per cent beryllium oxide, which was equivalent to 0.223 per cent beryllium. Martin ('33) stated that the United States Bureau of Mines had found comparable amounts of beryllium in the soil and that the beryllium was in the form of beryl. Beryllium was also detected in samples of soil from the Upper Peninsula, the College Farm and a sample of the Hillsdale type of soil.

The rat stock ration was composed of:

	parts		*parts*
Yellow corn	36	Alfalfa	5
Barley	30	Bone meal	2
Skim milk powder	10	Salt	1
Blood meal	5	Cod liver oil	1

The chemical analysis of the above ration was as follows:

	per cent		*per cent*
Moisture	9.30	Calcium	0.92
Protein	18.24	Phosphorus	0.67
Ash	5.71	Magnesium	0.16
Crude fiber	3.93	Fat	3.49

The blood was obtained from the chickens by heart puncture and pooled for each group. The blood from the dogs was also obtained by heart puncture. Lithium citrate was used as the anticoagulant. The plasma calcium and inorganic phosphorus values were determined by the Shohl ('22) modification of the Kramer-Tisdall method and the Briggs ('22) modification of the Bell-Doisy technic, respectively.

The right tibia of each chicken and the right humerus of each dog were cleaned, sawed into desirable lengths and extracted with hot 95 per cent alcohol for 72 hours. The fat-free, moisture-free bones were weighed into crucibles and ashed. After cooling in a desiccator the samples were reweighed and ground to pass a 20-mesh sieve. Aliquot portions of the ash were taken for the determination of calcium and phosphorus by the above-mentioned methods and the magnesium by a modification of the Briggs technic (Duncan, Huffman and Robinson, '35).

Rat feeding trials. Three groups of albino rats, each containing four animals approximately 28 days of age, were fed the regular stock rat ration which had been supplemented with, 1) 10 per cent of the soil, dried and ground to pass a 40-mesh sieve, 2) 20 per cent of the soil and 3) 0.25 per cent beryllium carbonate. The rats in groups 1 and 2 tolerated the soil well, grew normally and seemed to thrive on the diet. The rats in group 3 began to show rachitic symptoms within 30 days. Their gait became waddling, their backs were arched and the ankle joints were enlarged and stiff. The rats were definitely under normal weight and all of the rats in this group manifested rickets. The rats in the other two groups, however, were normal in weight and in appearance. After 40 days on experiment, the rats in groups 2 and 3 were killed and subjected to macroscopic examination. The rats in group 3 showed the typical rachitic symptoms. There were also indications of slight anemia. None of the symptoms observed in the rats rendered rachitic by beryllium carbonate were observed in the rats which had had 20 per cent of their diet supplemented with soil.

The soil intake was then increased to 20 per cent of the diet of the rats in group 1 with the object of studying the effect of feeding a high level of soil over a long period of time. The rats were allowed to mature on this diet, breed and reproduce. No changes were made in the diet of the pregnant or lactating mother except to add occasionally a few drops of cod liver oil. The litters were always reduced to six. After each of the female rats had produced one litter and had raised them to weaning age, the mothers and the male were destroyed. All of the offspring were kept on the experimental diet for 30 days after weaning. At that time, six female and one male rat were selected to be continued on experiment. The rats which were destroyed were always examined for rachitic symptoms. In the above manner, four successive generations of rats were raised on the regular stock diet supplemented with 20 per cent soil. The diet of the fifth generation was modified by increasing the soil intake to 30 per cent. Three generations of rats were raised on the high soil level. All of the rats were normal in every respect; they grew well and seemed to thrive on the diet.

The amount of beryllium in the 20 and 30 per cent soil diets was equivalent to 0.0446 and 0.0669 per cent, respectively, whereas the amount of beryllium in the 0.25 per cent beryllium carbonate diet was equivalent to 0.0329 per cent.

Chicken feeding trials. Eighty chickens, about 8 weeks of age, were divided into four approximately equal groups with respect to weight. Each group was fed a standard growing mash plus 2 per cent cod liver oil to compensate for laboratory conditions. In addition to the above ration, pen A had 5 per cent of the ration supplemented with soil which had been dried and ground to pass a 40-mesh sieve. Pens B and C each had 10 per cent of the ration supplemented with suspected soil from two sources. Pen D was the check group. The chickens were weighed at the beginning of the experiment and at regular intervals thereafter. The average percentage gains in weight of each group after being on experiment for 3 weeks are shown in table 1.

TABLE 1

Average initial weights and gains in weight of the chickens after 3 weeks on experiment

	INITIAL WEIGHT	FINAL WEIGHT	GAIN WEIGHT	GAIN
	gm.	*gm.*	*gm.*	*per cent*
Pen A	347	609	262	75.5
Pen B	335	580	245	73.2
Pen C	365	650	285	78.1
Pen D	411	670	259	63.0

The results of this phase of the experiment did not indicate any deleterious effects from the ingestion of soil so chickens in pen D were discarded and the rations were modified to increase the levels of soil intake. The chickens in pen A were designated pen E and the soil intake was increased to 25 per cent. The chickens in pens B and C received the same amount of soil as before but additional soil was kept before them at all times. Three chickens each from pens B and C were put into a separate pen and fed the soil as 50 per cent of the ration. These chickens were designated pen F. The average rate of growth of the chickens in each group is presented in table 2.

TABLE 2

Average weight of the chickens at the end of each period

	INITIAL WEIGHT	WEIGHT 3RD WEEK	WEIGHT 5TH WEEK	WEIGHT 7TH WEEK	WEIGHT 11TH WEEK	WEIGHT 3.5 MONTHS
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Pen B	335	580	813	991	1471	2178
Pen C	365	650	885	1012	1464	2018
Pen E	347	609	866	1018	1552	2262
Pen F		435	685	892	1353	1935

The most interesting observation that can be made from a study of table 2 is that the chickens which received a ration supplemented with 25 per cent soil made the largest gains, whereas the chickens which had 50 per cent of their ration supplemented with soil made almost as good gains. The chickens which had 10 per cent of their ration supplemented with soil and received soil ad lib. made intermediate but comparable gains.

The chickens were sacrificed after being on experiment for 3.5 months and the blood and bones were analyzed for their mineral content. The results are composited and summarized in table 3.

TABLE 3

Composition of bones and blood of chickens receiving various levels of soil (composite)

	RIGHT TIBIA				BLOOD PLASMA	
	Ash	Ca	P	Mg	Ca	P
	per cent	*per cent*	*per cent*	*per cent*	*milligrams per 100 cc.*	
10 per cent soil + soil ad lib.	58.00	22.35	10.10	0.60	12.3	6.68
10 per cent soil + soil ad lib.	58.40	22.13	10.26	0.83	9.1	7.23
25 per cent soil	55.35	21.43	9.64	0.59	11.2	6.72
50 per cent soil	54.50	20.81	9.55	0.77	11.5	6.88

The results from this experiment indicate that the feeding of soil to young chickens did not produce any observable ill effects due to the beryllium content or to the high levels of soil intake. Growth was not interfered with nor was the percentage composition of the tibia reduced because of the high intakes of soil.

Dog feeding trials. Since foxes were among the animals supposed to be affected by the soil, it was deemed advisable to test the soil on dogs. It was thought also that dogs would demonstrate any toxicosis due to the ingestion of soil better than any other species of animals because of the high hydrochloric acid content of their gastric secretions.

Four puppies which had been raised under the same conditions in regard to diet and general surroundings were brought to the laboratory and placed in a large cage immediately after weaning. The puppies were of mongrel breed, two being males and two females. Each puppy received a mixture of a commercially prepared dog meat and commercially prepared dog biscuit in amounts according to the needs of each animal. They also received 1 pint of milk and 1.0 cc. of cod liver oil each per day. Each puppy was fed in an individual cage. Dog 1 was the control and received the above unsupplemented ration. Thirty per cent of the ration, on the dry-matter basis, of dog 2 was supplemented with soil

which had been dried and ground to pass a 40-mesh sieve. Sixty per cent of the ration of dog 3 was supplemented with soil. The ration of dog 4 was supplemented with 0.75 gm. of beryllium carbonate, equivalent to 0.10 gm. of beryllium, per day, throughout the experiment. In the ration which contained 30 per cent soil, the actual beryllium intake varied from 0.045 gm. per day at the beginning of the experiment to 0.08 gm. per day when the experiment was completed. The 60 per cent soil ration varied from 0.09 gm. to 0.15 gm. per day of beryllium.

After about 15 days on experiment, dog 4 began to refuse the dog biscuits and after 30 days, the biscuits were refused at all times. At about this time dog 4 also began to refuse cold milk but when the milk was warmed to room temperature, it was readily consumed. The dog acted as though its mouth and teeth were sore and upon examination it was found that the teeth were less firmly embedded than normal. Dogs 1, 2 and 3 had ravenous appetites at all times. On several occasions, however, dog 3 would be off-feed in the morning or evening but would make up for it at the next feeding. The feeding experiments were continued for 3 months.

Table 4 shows the weight of each dog when placed on experiment and the rate of growth in body weight throughout the feeding trials.

TABLE 4

Changes in body weight of the four dogs

DATE	DOG 1	DOG 2	DOG 3	DOG 4
1934	*gm.*	*gm.*	*gm.*	*gm.*
6–19	1080	1625	1380	1690
6–26	1360	2130	1800	1955
7–11	1965	2900	2320	2130
7–25	2960	4180	3280	2210
8–10	3940	5290	4170	2110
8–25	4980	6845	5370	2090
9–17	6370	8195	6825	1880

The physical condition of dogs 1, 2 and 3 at the completion of the experiment was, to all outward appearances, good.

Their coats of hair were sleek, the teeth were normal and well developed, their legs were strong and straight and they were alert and playful. There were no evidences of rickets, abnormal cravings for foreign objects or physical deformities. Dog 4, however, grew slowly during the first 5 weeks on experiment and then progressively lost weight. The ankle joints became enlarged and the back became arched. The dog showed extreme emaciation in spite of the fact that the appetite for soft food was good. The dog was almost unable to stand unassisted at the termination of the experiment.

Table 5 presents some of the physical measurements of three dogs at the end of the experiment.

TABLE 5

Physical measurements of the experimental dogs

	DOG 1	DOG 3	DOG 4
	cm.	cm.	cm.
Length of head and neck	14	16	15
Length of body	42	43	28
Length of tail	23	25	16
Length of front legs	17	17	12
Height at front shoulders	39	41	20
Height at rear quarters	36	39	24

The data in table 5 demonstrate the stunting effect upon the growth of dog 4 due to the ingestion of beryllium carbonate. This effect was not noted in dog 3 which was fed the soil as 60 per cent of the ration and equivalent to 0.09 to 0.15 gm. of beryllium per day.

Table 6 gives the concentrations of calcium and inorganic phosphorus in the blood plasma and the ash and mineral content of the right humerus of each dog.

TABLE 6

Concentrations of blood plasma calcium and inorganic phosphorus and the ash and mineral content of the humeri

	BLOOD PLASMA		RIGHT HUMERUS			
	Ca	P	Ash	Ca	P	Mg
	milligrams per 100 cc.		*per cent*	*per cent*	*per cent*	*per cent*
Dog 1	13.1	4.77	56.45	21.22	10.02	0.84
Dog 3	13.1	8.23	54.45	20.90	9.62	1.26
Dog 4	12.6	2.44	55.40	21.93	9.46	0.55

It will be noted that the plasma calcium and inorganic phosphorus were normal in dogs 1 and 3. The calcium was normal in dog 4 but the inorganic phosphorus was markedly subnormal. The ash and mineral content of the humeri of all of the dogs were normal and showed good agreement. It is well known that growth is an important factor in the development of rickets, the greater the rate of growth, the more marked the tendency to develop rickets in animals. The lack of growth works strongly against the induction of rickets and is well illustrated in the ash and mineral content of the humerus of dog 4.

Post-mortem examination was made of each dog. Dogs 1 and 3 were in good condition of flesh and moderately fat. All of the internal organs were normal in color. There was no evidence of anemia. Dog 4 was extremely emaciated and the internal organs were lighter in color than normal because of slight anemia. The heart and gall bladder were unusually large for the size of the dog. There was one yellow gelatinous mass, about 1 cm. in diameter, on the liver. The ribs showed marked rachitic beading. There were no evidences of decayed or malnourished teeth, although the dog's mouth had been sensitive to hard food and cold liquids.

DISCUSSION

Seven successive generations of rats were raised which had their diet supplemented with the suspected soil. The first four generations had 20 per cent of their diet replaced by the soil which was equivalent to the ingestion of 0.0446 per cent beryllium. The diet of the fifth generation was increased to 30 per cent and the two succeeding generations were raised to maturity on the higher level. The ingestion of this amount of soil was equivalent to 0.0669 per cent beryllium. The ingestion of a small amount of beryllium carbonate (0.0329 per cent beryllium) with an otherwise adequate diet resulted in the production of rachitic symptoms in rats in 20 to 30 days.

The young rats were placed on the experimental diet at weaning age because young rats are more susceptible to a rachitogenic regime. This procedure proved ineffective since the beryllium contained in the soil was unavailable to the animal body. The results from long-time observations with rats indicate, however, that these animals can tolerate unusually large amounts of soil in their diet.

The experiment with young chickens did not indicate any deleterious effects when the soil was fed up to 50 per cent of the ration. Growth was not interfered with and the bone ash determinations did not indicate poor utilization of calcium and phosphorus due to the ingestion of large amounts of soil.

The experiment with dogs indicated that intakes of soil up to 60 per cent of the ration were well tolerated. The dog on the high soil ration refused his feed on several occasions but this did not result in poor growth, a mineral deficiency or malnutrition. There were no unfavorable results due to the beryllium content or to the high levels of soil intake. The dog which received a small amount of beryllium carbonate soon developed all of the outward symptoms of rickets.

One of the chemical properties of beryllium is the formation of a phosphate which is exceedingly insoluble, even in moderately acid solutions. Any ingested soluble beryllium salt would be decomposed and the beryllium quantitatively precipitated as the phosphate by any free phosphate present in the intestinal tract. This insoluble precipitate would be excreted and thereby cause a constant drain on the animals' intake of phosphorus. In view of the fact that the beryllium compound present in the soil is extremely insoluble in all ordinary reagents, it was, therefore, impossible to get enough beryllium into solution in the gastric or intestinal secretions of the experimental animals to cause a serious phosphorus deficiency.

These experiments failed to produce any bone lesions which could be associated with rickets or to the ingestion of a radioactive substance. These results do not substantiate the belief of Hanna ('33) that beryllium has distinct radioactive properties.

Evvard and associates ('27) found that the feeding of black loam soil to 2-year-old feeder steers resulted in greater body gains, a lower cost of gain and considerably more internal fat than for a corresponding lot of steers which received the same basal ration minus the soil.

The results of these experiments also confirm the finding of Weinert ('35) who reported that the feeding of quartz sand up to 31.5 per cent of the ration had no marked influence on the digestive function of wethers, that sand in amounts up to 30 per cent or dirt up to 25 per cent of the ration had no marked influence upon milk production in cows over 10-day periods, and that a diet containing 50 per cent sand did not affect the appetite or the digestive function of growing rats.

SUMMARY AND CONCLUSIONS

Feeding experiments with rats, chickens and dogs have been conducted to investigate the possibility of the occurrence of a natural beryllium toxicosis due to the ingestion of soil.

The feeding of a diet which contained 30 per cent soil to rats was well tolerated and did not predispose them to any of the rachitic symptoms produced in the rats which received 0.25 per cent beryllium carbonate in their diet.

Chickens tolerated up to 50 per cent of the soil in their ration. The beryllium in the soil failed to interfere with normal growth or to reduce the concentration of inorganic phosphorus in the blood plasma.

The ingestion of a ration which contained 60 per cent soil was well tolerated and did not result in poor growth in dogs, nor did it produce a phosphorus deficiency due to the beryllium in the soil.

These results fail to verify the report that the ingestion of soil in certain counties in northern Michigan produced malnourished and deformed animals. The amount of soil fed to the animals in these experiments exceeded any amount that the animal would naturally consume when receiving a well-balanced ration.

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THE VITAMIN CONTENT OF CANNED PINEAPPLE JUICE

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INTRODUCTION

The juices of certain fruits and vegetables have become exceedingly popular as constituents of the human dietary. This increased popularity is apparently due to a combination of qualities or factors, such as palatability, nutritional value, ease of serving, etc. A juice which has received favorable consideration during the past year is that of the pineapple. As to the nutritive value of commercially canned pineapple juice, especially concerning its vitamin content, little can be said, because of the lack of definite scientific data. Data are available, however, which show that both fresh and canned pineapple are good sources of several of the vitamins and, in addition, contain other highly desirable nutritive properties. The investigations of Miller ('24) and Killian ('32) are referred to in this connection.

Since the data presented by the above investigators indicate that both fresh and canned pineapple are good sources of several of the vitamins, it seemed that similar data concerning the vitamin content of commercially canned pineapple juice would have a definite value at this time. The studies herein reported were carried out for the purpose of determining the vitamin A, B, C and G content of this juice.

EXPERIMENTAL

Vitamin A. The method used in the determination of the vitamin A potency of the pineapple juice was essentially that of Sherman and Munsell ('25). Young rats, 20 to 21 days of age and weighing between 40 and 46 gm., were used as the experimental animals. At weaning, each rat was placed in an individual metal cage having a raised screen floor, and was supplied with fresh distilled water and a liberal quantity of the basal (vitamin A-free) diet. An allotment of 0.6 gm. of dry irradiated baker's yeast was fed daily as a source of vitamins B, D and G. All animals were weighed at frequent intervals, at which time a record was made as to the amount of food consumed and the general well-being of the animals. When the animals had been depleted of their body stores of vitamin A, as indicated by cessation of growth and the appearance of incipient xerophthalmia, they were arranged in groups of from six to ten animals, at which point each animal was fed daily an accurately measured quantity of the pineapple juice as the supplement to the vitamin A-free diet. In every case, this supplementary feeding of pineapple juice was continued for a period of at least 6 weeks before the experiments were terminated.

The results obtained through this series of experiments are presented in condensed form in table 1.

The data presented in table 1 indicate that 1 ml. of this juice contains approximately 1 Sherman unit of vitamin A. On the basis of this assay, therefore, the canned pineapple juice tested contained approximately 30 Sherman units of vitamin A per ounce.

Vitamin B (B_1). The Chase and Sherman ('31) method was used in determining the vitamin B potency of the canned pineapple juice. The experimental animals and their care and management were essentially the same as that employed in the vitamin A assays, excepting that a vitamin B deficient diet was employed. When the animals had been depleted of their body reserves, as indicated by cessation of growth and the appearance of mild symptoms of paralysis, they were

arranged in groups of from six to ten animals each. A measured amount of the juice to be tested was fed daily in a separate receptacle to each animal. The feeding of the juice was continued for a period of 6 weeks, during which time an accurate record was kept of each animal's weight, the amount of food consumed, and the general appearance of the animal.

TABLE 1

Showing the effect on growth of feeding different daily allotments of pineapple juice to groups of rats which were receiving a diet deficient in vitamin A. For sake of comparison a similar group of rats was fed 1 γ of International carotene daily as a source of vitamin A

Group	Average initial weight	Depletion period: Average weight at end of 7 days	14 days	21 days	28 days	Depletion period	Source of vitamin A, quantity fed daily	Curative period: Average weight at end of 7 days	14 days	21 days	28 days	35 days	42 days	Gain in weight
	gm.	gm.	gm.	gm.	gm.	gm.		gm.	gm.	gm.	gm.	gm.	gm.	gm.
1	42	64	81	107	123	129	None	127	122	115	...	...	...	..
2	45	70	86	106	134	138	1.0 ml. of pineapple juice	147	154	159	159	159	161	23
3	44	68	85	100	126	132	1.5 ml. of pineapple juice	142	147	154	158	161	162	30
4	44	68	91	116	130	135	2.0 ml. of pineapple juice	143	149	158	163	165	169	34
5	45	67	89	111	131	134	3.0 ml. of pineapple juice	143	150	159	165	169	176	42
6	44	68	87	116	131	138	4.0 ml. of pineapple juice	149	161	172	181	191	196	58
7	45	67	93	114	132	136	5.0 ml. of pineapple juice	143	155	169	180	187	198	62
8	41	61	80	103	118	123	1 γ International carotene	130	139	143	149	153	158	35

The results obtained in this series of experiments are presented in condensed form in table 2.

From these results it is evident that 1.5 ml. of this canned pineapple juice contained at least 1 Sherman unit of vitamin B. On this basis, 1 ounce of this juice contained approximately 20 Sherman units of this vitamin.

Vitamin G (B_2). The method used in determining the vitamin G potency of the canned pineapple juice was that

described by Bourquin and Sherman ('31). Here again young rats, 20 to 21 days of age and weighing between 40 and 46 gm., were used as the experimental animals. The care and treatment of the experimental animals was the same as that reported under vitamin A. After the test animals had been partially depleted of their body stores of vitamin G,

TABLE 2

Showing the growth stimulating effect of pineapple juice when fed at different levels to groups of rats which were receiving a diet deficient in vitamin B. For sake of comparison a similar group of rats received 10 mg. of an International vitamin B preparation

DEPLETION PERIOD						CURATIVE PERIOD						
		Average weight at end of				Average weight at end of						
Group	Average initial weight	7 days	14 days	Depletion period	SOURCE OF VITAMIN B. QUANTITY FED DAILY	7 days	14 days	21 days	28 days	35 days	42 days	Gain in weight
	gm.	gm.	gm.	gm.		gm.	gm.	gm.	gm.	gm.	gm.	gm.
1	42	53	58	64	None	60	49	40	..	..	..	..
2	45	59	64	66	1.0 ml. of pineapple juice	68	69	70	72	73	70	4
3	42	56	63	62	1.5 ml. of pineapple juice	68	71	73	75	81	85	23
4	45	55	65	64	2.0 ml. of pineapple juice	67	74	80	85	89	90	26
5	42	52	63	60	3.0 ml. of pineapple juice	64	76	82	84	92	96	36
6	43	53	63	64	4.0 ml. of pineapple juice	71	83	95	101	114	118	54
7	41	49	62	61	10 mg. of International vitamin B preparation	72	77	84	91	98	102	41

as indicated by a definite decrease in their growth rate, they were arranged in groups of from six to ten animals per group and were fed daily measured quantities of the pineapple juice. The feeding of this juice to groups of rats as a supplement to a vitamin G deficient diet was continued for a period of 8 weeks, during which all observations were accurately recorded.

The results obtained by this series of experiments are presented in table 3.

The data presented above indicate that a daily allotment of 12 ml. of the canned pineapple juice furnished slightly more than 1 Sherman unit of this vitamin. According to this assay, each ounce of this juice contained approximately 2.5 Sherman units of vitamin G.

TABLE 3

Showing the growth stimulating effect of pineapple juice when fed at different levels to groups of rats which were receiving a diet deficient in vitamin G.

DEPLETION PERIOD						CURATIVE PERIOD								
		Average weight at end of				Average weight at end of								
Group	Average initial weight	7 days	14 days	Depletion period	SOURCE OF VITAMIN G. QUANTITY FED DAILY	7 days	14 days	21 days	28 days	35 days	42 days	49 days	56 days	Gain in weight
	gm.	*gm.*	*gm.*	*gm.*		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	43	47	53	54	None	56	58	55	53	51	51	49	46	—8
2	42	49	53	53	4.0 ml. of pineapple juice	55	55	56	56	59	59	55	56	3
3	44	53	57	57	6.0 ml. of pineapple juice	63	63	65	64	65	64	63	62	5
4	42	50	52	51	8.0 ml. of pineapple juice	56	54	54	55	55	55	54	55	4
5	44	53	55	56	10.0 ml. of pineapple juice	63	65	64	66	66	68	65	66	10
6	44	51	54	53	12.0 ml. of pineapple juice	62	65	67	68	73	74	76	77	24
7	42	49	53	52	14.0 ml. of pineapple juice	60	64	66	67	69	71	76	81	29

Vitamin C. The vitamin C content of the canned pineapple juice was determined according to the prophylactic technic of Sherman, La Mer and Campbell ('22). Young guinea pigs, ranging in weight from 250 to 300 gm. each, were kept in individual metal cages, which were provided with raised screen bottoms. These animals were arranged in groups of from six to ten individuals per group, and each guinea pig was provided with a weighed but liberal quantity of the scurvy producing diet. Fresh distilled water was provided daily for all animals. Each animal was weighed at weekly

intervals and an accurate record was made of the amount of food consumed. At each weighing period, all residual food remaining from the previous week was discarded and a freshly prepared portion fed.

A supply of the pineapple juice sufficient for 1 week was kept in the refrigerator at all times, and the cans were opened just prior to the feeding of the juice. Starting with the first day of the experimental period, an accurately measured amount of pineapple juice, in a clean earthenware container, was fed daily to each pig. The animals comprising each of the several groups received identical allotments of the juice under test. The duration of the test period ranged from 56 to 90 days. When it became definitely evident that certain groups of pigs were receiving pineapple juice in great excess of that required to protect them against scurvy, such groups of animals were discontinued. Those receiving the higher levels of juice (10 ml. daily) were discontinued after 56 days, while those that received slightly less juice (9 ml. daily) were discontinued at the end of 70 days. All animals that received less than a protective dose of the juice and those that received a protective dose or slightly more than a protective dose were continued on experiment until death ensued or throughout the 90-day period. All animals that died and those that were killed at the termination of the various experiments were autopsied and rated according to the various degrees of scurvy manifested (Sherman, La Mer and Campbell, '22). These various degrees of scurvy (viz., hemorrhage of rib junction, muscles, joints, and intestines; fragility of the jaw bone and of the joints; looseness of the teeth; beading of the rib junctions) were scored from 0 to 3, according to the degree of scurvy manifested by each tissue. According to this method of scoring, a score of 8 indicated definite scurvy, while a score of 24 indicated the highest possible degree of scurvy. The quantity of juice required to produce a score of from 0 to 2 was considered the minimum protective dose.

The data obtained from this series of experiments are presented in table 4. It will be noted that all animals of groups 1 and 2 died during the course of the experiment. It may be of interest to state in this connection that all animals of group 1 were dead by the thirty-first day, while all animals of group 2 were dead by the forty-second day.

From the data presented in table 4, it is evident that canned pineapple juice contains appreciable quantities of vitamin C.

TABLE 4

Showing the results obtained by feeding varying amounts of canned pineapple juice to groups of young guinea pigs that were receiving a scurvy-producing diet

ANIMAL GROUP NO.	NUMBER OF ANIMALS CONSIDERED	QUANTITY OF JUICE FED DAILY	AVERAGE DAILY GAIN IN WEIGHT	AVERAGE SCURVY SCORE
		ml.	*gm.*	
1	6	0	All died	Severe
2	6	3.0	All died	Severe
3	3	4.0	0	17
4	4	5.0	0.5	8
5	9	6.0	1.8	3
6	7	7.0	2.4	0–2
7	5	8.0	2.7	0
8	8	9.0	2.7	0
9	7	10.0	2.9	0

Approximately 7 ml. of this juice were required to furnish a minimum protective dose of this vitamin. From this, it follows that the canned pineapple juice tested contained approximately four minimum protective doses of vitamin C per ounce. Since a minimum protective dose of vitamin C is approximately 10 International units of this vitamin, it would follow from the above scurvy scores that 1 ounce of canned pineapple juice contained approximately 40 International units of vitamin C.

SUMMARY

A biological assay of canned pineapple juice for its vitamin content, using standard biological methods, showed this juice to be a good source of vitamins A and B, a fair source of vitamin C, and to contain a measurable quantity of vitamin G. When expressed in terms of vitamin units, 1 ounce of this juice contained approximately 30 Sherman units of vitamin A, 20 Sherman units of vitamin B, 2.5 Sherman units of vitamin G, and four minimum protective doses or 40 International units of vitamin C.

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